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POST GRADUATE PROGRAMS

DIVERSITY AND SYMBIOTIC EFFECTIVENESS OF *RHIZOBIUM* ISOLATES COLLECTED FROM DIFFERENT FABA BEAN (*VICIA FABA*) GROWING AREAS OF NORTH AND SOUTH GONDAR, ETHIOPIA:

MSc. Thesis

By

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LIST OF ABBREVIATIONS

%	percent
µg	micro gram
µl	micro liter
$_{15}N$	fifteen nitrogen method
ATP	Adenosine triphosphate
BNF	Biological Nitrogen Fixation
BTB	Bromothymol-blue
CHI	Chilga
cm	centimeter
CSA	central statistical agency
DM	Dry mass
FAOSTAT	Statistical database of the Food and Agricultural Organization
ha	hectare
HC	hydrochloric acid
hr	hour
ICARDA	International Center for Agricultural Research in the Dry Areas
KD	Kimir Dingay
Kg	kilogram
Km	kilo meter
LG	Lay Gayint
LHb	leghaemoglobin
M	Molar
M.a.s.l	meters above sea level
min	minute

ml	milliliter
mM	milliMolar
MT	metric tons
N	Normality
N_2	dinitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
^oC	degree centigrade
Ppm	Parts per million
TCA	tricyclic acid
UPGMA	unweighted pair group method with the average
v/v	volume by volume
w/v	weight by volume
YEMA/B	Yeast Extract Mannitol agar/broth

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ABSTRACT

Biological nitrogen fixation is a natural process for fixation of molecular nitrogen to maintain sustainable agriculture throughout the world. Thus, the aim of this study was to isolate, characterize and select potential *Rhizobium* strains collected from different faba bean growing areas of North and South Gondar, Ethiopia. About 3kg soil samples from each site with previous history of faba bean cultivation was collected and brought to the laboratory for further analysis. A total of 57 *Rhizobium* isolates were isolated based on infection method. The isolates were characterized on Yeast Extract Mannitol Agar (YEMA) medium and authentication of strain test was done on sand culture using pot experiment. The isolates attain colony sizes ranging from 1.5 to 4.5 mm after 3 to 5 days of incubation. All the isolates showed growth on all the tested carbon sources except lactose, arabinose and mannose. Most of the isolates showed growth at different pH levels ranging 5 to 9, salt concentrations from 0.1% to 2% (w/v), temperatures between 5 and 45°C and at different antibiotics with different concentrations. In sand culture, only 35.5% of isolates showed nodulation. Analyses of variance indicated, inoculation of isolates improves tested traits significantly ($p< 0.05$) at all measured investigated parameters such as shoot length, shoot dry weight, and plant total nitrogen as 11%, 28% and 31.3%, respectively, over nitrogen treated plants, 2%, 10% and 29.4% respectively over standard *Rhizobium* and 55%, 82.3% and 85.7% respectively over negative treatments. Shoot dry weight was found to be strongly positively correlated with symbiotic effectiveness ($r= 0.994$, $P <0.01$). Based on symbiotic effectiveness, 80% of the tested isolates was found to be highly effective, 13% effective and only one isolate less effective. From this finding, most of our isolates displayed abundant diversity in their response to morphological and physiological characteristics. Inoculation of selected *Rhizobium* isolates revealed shoot dry weight enhancement over nitrogen treated plants of faba bean on sand culture using pot experiment under controlled greenhouse condition. Dendrogram analysis shows that isolates were categorized into six major clusters that were again sub clustered to many. Best isolates for various agro-climatic regions were identified. Therefore, there is a need for detail study of effective isolates tested on field condition and molecular characterization for better classification of the *Rhizobium* isolates.

Key words: Nitrogen fixation, *Rhizobium*, Total nitrogen, Authentication, Dendrogramanalysis

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1. INTRODUCTION

Ethiopia is a home to about a dozen species of tropical grain legumes. An estimated 1.5 million ha of land is planted to grain legumes in this country and more than 1.9 million metric tons (MT) of grain produced each year, touching the lives of about 10 million households(Ronner*et al*., 2012). Grain legumes have a nitrogen fixing symbiosis with soil root nodule bacteria (Vance, 1997). Among these, Faba bean (*Vicia faba* L.) is an annual grain legume widely cultivated, which serves as foods for human and animal nutrition in many countries, since it is rich in protein, minerals and vitamins (Sillero *et al*., 2010).Cultivation of faba bean plays important roles in maintaining sustainable agriculture system in many marginal areas; due to its high nutritional value, multiple uses and ability to grow over a wide range of climatic and soil conditions (Nadal *et al*., 2003).

Faba bean, considered to be one of the nine founder crops (or primary domesticates), is probably native to southwestern Asia (Tanno and Willcox, 2006; Zeder, 2009). After its domestication at least 7000 years ago, faba bean spread to Africa, the Mediterranean basin and central Europe, the Americas, China, India, etc. (Ye *et al*., 2003; Duc *et al*., 2010). China and Europe are the main producers, contributing 43.0% and 20%, respectively, of the world faba bean production (FAOSTAT, 2008).

Ethiopia is one of the largest faba bean producing countries in the world only second to China (Biruk, 2009)). Historically, Ethiopia is considered as the secondary center of diversity and also one of the nine major agro-geographical production regions of faba bean (Asfaw *et al*., 1994). Faba bean (*Vicial faba L*.) is the leading grain legume crop grown as field crop, a daily food and cash crop throughout the highlands and is most common in Wayena Dega between the altitudes 1800 and 3000 maslin Ethiopia (ICARDA, 2006; Temesgen and Aemiro, 2012).

Legumes are able to establish nitrogen-fixing symbioses with bacterial microsymbionts (rhizobia), thus reducing theneed for chemical fertilizers.This reduction may help to minimize greenhouse- gas emissions and to avoid contamination of ecosystems (Snyder *et al*., 2009). This association further provides a nitrogen supplement for the subsequent crops (Alexander *et al*., 2009). Faba bean, common bean, chickpea, and field pea combined account for about two-thirds of area planted to all legumes in Ethiopia. Faba bean (*Vicia faba*), field pea (*Pisum sativum*) and

lentil (*Lens culinaris*) together with chick pea (*Cicer arietinum*) are the major highland pulse crops in Ethiopia and occupy 12 to 15% of the land under cultivation (CSA, 2004; ICARDA, 2012). These leguminous crops are known for their ability to form endosymbiotic relationship with the root nodule bacteria collectively called Rhizobia.Faba bean like other legumes crops has the ability to form symbiotic association with root nodulating bacteria (rhizobia) group called *Rhizobium leguminosarum bv. Vicia*(Jordan, 2005).Faba bean mainly grown for its high protein content which accounts about 30 % on average and it is vital nutrient for human and animals as source of protein(Kamal *et al*., 2012). Faba bean popularity has increased recently as its high yield makes it attractive to producers while its high protein content and low-priced makes it attractive to consumers (Pala *et al*., 2000).

Biological Nitrogen Fixation (BNF) is a natural process of significant importance in world sustainable agriculture.Notably, the symbiotic relationship between rhizobia andlegumes annually produces around 60% of the total BNFinputs in world agriculture (Herridge *et al*., 2008). In most rhizobium–legumeinteractions, the symbiosis is initiated by a moleculardialogue in which plant flavonoids interact with rhizobial*NodD* proteins to activate the production of Nod factorsignals by the rhizobia (Cooper, 2004).The *Rhizobium*–legume symbioses vary in specificity forboth the breadth of host range and the diversity of bacterialspecies nodulating a given host plant. The interactionbetween the tribe *Viciae* (*Vicia, Pisum, Lens* and *Lathyrus*)and*Rhizobium leguminosarum bv. Viciae* was thoughtto be one of the most specific. Variations in the selection of*Viciae* species for specific *Rhizobium* genotypes have been documented, although different host species share symbionts (Alvarez *et al*., 2009).

The continual loss of nitrogen from the reserve of combined or fixed nitrogen, which is present in soil and available for use by plants impose limitations to the growth of green plants. Several processes continually deplete it, mainly by the removal of nitrogen containing crop residues from the land, microbial denitrification, soil erosion,leaching and chemical volatilization. Hence the nitrogen reserve of agricultural soils needs to be replenished periodically in order to maintain an adequate level for crop production.This replacement of soil nitrogen is accomplished by the addition of chemically fixed nitrogen in the form of commercial inorganic fertilizers or by the activity of biological nitrogen fixation systems (Neamat and Raffal, 2013). The symbiotic association between rhizobia and legumes plays a significant role in the world agricultural

productivity by annually converting approximately 120 million tons of atmospheric nitrogen into ammonia thereby saving US$ 6.8 billion expenditure on nitrogenous fertilizer (Herridge and Rose, 2000).

Identification and characterization of root nodule bacteria has paramount important, due to various reasons, (i) it provides a valuable resource for selecting efficient and competitive strains tolerant to a variety of edaphic factors (Neves and Rumjanek, 1997), (ii) their symbiosis have major environmental and agricultural importance since they are responsible for most of the biological atmospheric nitrogen fixation on earth (Zakhia and Lajude, 2001), (iii) to evaluate competitiveness and the effects of specific inoculant strains on the growth of leguminous plants (Neamat and Raffal, 2013).

In Ethiopia, for the last three decades attempts have been made to conduct research on cool season legumes. The major areas of research conducted so far include nodule collections and inoculation needs of these pulse crops. Identification using a few phenotypic characters were undertaken to identify several isolates of *Rhizobium leguminosarumbv.vicieae* (Alemayehu, 2009; Zerihun and Fassil, 2011) from few regions of Ethiopia. However, reports on rhizobial polyphasic characterization approach and selection of potential *Rhizobium leguminosarum bv. viciae*that show superior in nitrogen fixing capacity are still insufficient. Hence, the present study aims to identify efficient *Rhizobium*isolates and their effect on nodulation isolated from the major faba bean producing areas of different districts of North and South Gondar using polyphasic characterization approach.

1.1. Statement of the Problem

Many research works of different scholars has indicated theenvironmental symbiotic association of *Rhizobium* and legumes for fixation of atmospheric dinitrogen in the world in different countries. Legumes plays a significance role in maintaining productivity in agricultural systems, serves as foods for human and animal nutrition and sources of income in both developed and developing countries. Legumes are rich in protein, minerals and vitamins content.However, in Ethiopia so far, only very little information is available about biological nitrogen fixation to maintain legume yields in agriculture system. In Ethiopia small scale farmers do not have the practice of using organic fertilizers and rotation of legumes (particularly faba bean) on routine basis. The application of inorganic nitrogen fertilizers is commonly used by the farmers in Ethiopia. However, these types of fertilizers are expensive, have limited distribution for farmers residing in remote rural parts of the country and have huge environmental impact such as global warming, deterioration of water bodies and soil infertility. Therefore, the present study is proposed to alleviate the existing problems by identifying the potential Rhizobium strains collected from different faba bean growing areas of North and South Gondar to be used as inoculants.

2. OBJECTIVES

2.1. General Objective:

- ✓ To examine the diversity and symbiotic effectiveness of *Rhizobiumleguminosarum bv. viciae* isolates collected from different Faba bean (*Vicia faba*) growing areas of North and South Gondar zones.

2.2. Specific objectives:

The specific objectives of this study are to:

- ✓ Isolate *Rhizobium* strains collected from different locations of South and North Gondar.
- ✓ Characterize different isolates of *Rhizobium* using morphological features, biochemical tests and physiological tests.
- ✓ Evaluate the symbiotic effectiveness of *Rhizobium* isolates under greenhouse condations using pot experiment.
- ✓ Select the potential *Rhizobium* strains that show superior in their nitrogen fixation capacity.

3. LITERATURE REVIEW

3.1. Nitrogen fixation process

Nitrogen is not a scarce element on earth but it is the most abundant forms of atmospheric dinitrogen (N_2) and is not directly available for plants. As a result, nitrogen is often a critical limiting element for plant growth and development in agriculture production. Nitrogen is a vital element in plant growth which is usually absorbed as nitrate or ammonium, taking part in major components of proteins, enzymes and chlorophyll, the most important pigment needed for photosynthesis (Malakooti and Tabatabayee, 2005). It is also found in other important biomolecules, such as ATP and nucleic acids. Even though it is one of the most abundant elements (predominately in the form of nitrogen gas (N2) in the Earth's atmosphere), plants can only utilize reduced forms of this element. Plants acquire these forms of "combined" nitrogen through fixation process in : i) the addition of ammonia and/or nitrate fertilizer (from the Haber-Bosch process) to soil, ii) the release of these compounds during organic matter decomposition, iii) the conversion of atmospheric nitrogen into the compounds by natural processes, such as lightning, and iv) biological nitrogen fixation (Vance, 2001).

3.2. Biological Nitrogen-fixation

Nitrogen is provided to agricultural lands by the application of urea and ammonium nitrate chemical fertilizers, but the utilization of inorganic fertilizers are not ecofriendly for environment, which leads to pollution of water bodies, air and soil. The harmful effects of these chemical inputs have encouraged researchers to develop the sustainable agriculture practices, for example by applying the biological fertilizers (Samavat *et al.*, 2012). According to Samavat *et al*., 2012*Rhizobium* inoculum is a bio fertilizer, which meets some part of legumes nitrogen requirement through the biological nitrogen fixation. This biological nitrogen fixation system can fix 70 - 85 million ton of nitrogen annually, which is about 50% of the world fixed nitrogen, and equivalent to all chemical fertilizer factories production (Saleh-Rastin, 1992; Afshari, 1996). Biological nitrogen fixation is a natural process that changes inert atmosphere nitrogen into biologically useful ammonia used by legumes (Smil, 2001).Based on SØrensen and Sessitsch (2007) the process is mediated in nature by nitrogen-fixing *rhizobia* bacteria (Rhizobiaceae, α-Proteobacteria).

Biological nitrogen fixation, the major means of recycling of nitrogen in the biosphere, is an economically justifiable, ecologically safe to develop sustainable agricultural production and building up soil fertility (Hardarson, 1993). It is a relatively inexpensive source of nitrogen for small-holder farmers in developing countries where chemical nitrogen input is neither available nor affordable, since the price of mineral fertilisers has tremendously increased and reached to the level that a good proportion of the subsistence farmers often face difficulty to purchase and utilize (Amanuel*et al*., 2000). Tate III (2000) found that symbiotic nitrogen fixation by legume-Rhizobium association is the major and well-exploited biological nitrogen fixation in soil based ecosystems, supplying about 64% of the biologically fixed nitrogen in terrestrial agricultural systems.

3.3. Biochemistry of biological nitrogen fixation

Fixation and reduction of atmospheric dinitrogen into ammonia is a sophisticated process which requires a huge amount of energy (Postgate, 1982). Since the molecule consists of two nitrogen atoms joined by a triple covalent bond which becomes chemically inert and nonreactive (Wagner, 2012). Nodules are specialized organs in legumes where symbiotic nitrogen fixation takes place, resulted from rhizobial infection (Krusell *et al*., 2005). The enzyme complex nitrogenase comprising the Fe and Mo subunits is responsible for the fixation of nitrogenase in root nodules. Sucrose, fructose and glucose are photosynthetic product which provides ATP to the nitrogen fixing system through the mechanism of oxidative phosphorylation in root nodule (Dommergues and Krupa, 1978). But nitrogenase enzyme is highly sensitivity towards oxygen thus requiring an oxygen protecting system (Downie, 2005).

White *et al*. (2007) found that leghaemoglobin (LHb) is oxygen binding protein which acts as an oxygen buffer that cycle between oxidized Fe3+ and reduced Fe2+ and helps to keep up oxygen level sufficiently low inside the nodule. Bacteroid is swollen structure with in plant cell formed by nitrogen fixing microorganisms such as *Rhizobium* and forms symbiosome when portion of plant cell membrane surrounds the bacteroids(Krusell *et al*., 2005). LHb lies within the membrane envelope that surrounds the Bacteroids and process of nitrogen fixation begins only after formation of symbiosome, since membrane envelopes are the site of primary reaction of nitrogen fixation. Photosynthetic products particularly, sucrose serves as the real substrate that support nitrogen fixation by bacteria. Trainer and Charles(2006) examined that hydroxybutyrate

dehydrogenase present in bacteria helps to generate the reducing power of ATP for the support of nitrogen fixation. TCA cycle is being the active pathway for pyruvate utilization and Resendis-Antonio *et al*.(2007) reveals that oxidative phosphorylation is the major source of ATP for nitrogen fixation. According to Lodwig and Poole(2003) finding major organic compounds that are transported across the symbiosome membrane and into the bacteroid are the intermediates of TCA cycle such as succinate, malate and fumarate.

3.4. Significance of biological nitrogen fixation to soil fertility

Agricultural soils replenished periodically in order to maintain an adequate level for crop production through mineral fertilizer or biological nitrogen fixation systems. The use of mineral nitrogen fertilizer source employed for decades and has a big impact in the environment. Biological nitrogen fixation is the major means of recycling of nitrogen in the biosphere, is an economically justifiable and ecologically safe nitrogen source to agriculture and plays a key role in land remediation (Peoples, 1995). There is severe of land degradation worldwide, and it is time to stop the destructive uses of land and to institute a serious reversal of land degradation (Burris, 1994). Based on Amanuel *et al*. (2000), biological nitrogen fixation is relatively low-cost source of nitrogen for small-holder farmers in developing countries where chemical nitrogen input is neither available nor affordable. It is important to develop sustainable agricultural production and building up soil fertility (Hardarson, 1993). Legume-*Rhizobium* symbiotic associations are very important for both ecologically and agriculturally in soil based ecosystems (Tate III, 2000) supplying about 64% of the biologically fixed nitrogen in terrestrial agricultural systems.

Studies reveal that biological nitrogen fixation is focused on the symbiotic system of leguminous plants and rhizobia, because these associations have the greatest quantitative impact on the nitrogen cycle. Among the legumes, such as common bean, faba bean, pea, chick pea, peanut and soybean (Brockwell *et al*., 1995; Peoples, 1995; Tate, 1995), this has a tremendous potential for contribution of fixation of nitrogen to soil ecosystems. Approximately, there are 700 genera and 13,000 species of legumes, but only about 20% (Sprentand Sprent, 1990) of the legumes have been tested for nodulation effectiveness and shown to have the ability to fix N_2.Legume symbioses contribute at least 70 million tons of nitrogen per year (Brockwell *et al*., 1995). Increased nutrition value of plants (protein in legumes) and reduced depletion of soil

nitrogenreserves are obvious consequences of legume N_2 fixation. Deprived of mineral nitrogen in the soil often reduce plant growth and development, and so symbiotic relationship can be take place between a variety of nitrogen-fixing organisms and plants (Freiberg *et al*., 1997).

For incorporation of biological nitrogen fixation into soil ecosystems, successful Rhizobium-legume symbioses will be mandatory. Association of Rhizobium-legume symbioses can fix Atmospheric N_2 into ammonia, nitrate and other organic fertilizer, which represents renewable source of nitrogen for agriculture (Peoples *et al*., 1995). Values estimated for various legume crops and pasture species are often impressive, commonly falling in the range of 200 to 300 kg of nitrogen $ha^{-1}year^{-1}$ (Peoples *et al*., 1995). The amounts of nitrogen fixed by symbiotic association in legumes and *Rhizobium* may be different according to the method used to study N_2 fixation (Sellstedt *et al*., 1993). Inputs of BNF into terrestrial ecosystems from the symbiotic relationship between legumes and *Rhizobium* accounts at least 70 million tons of nitrogen per year (Brockwell *et al*., 1995), this enormous quantity will enhance agricultural production as the world's population increases and substitute or diminish the use of inorganic nitrogen fertilizer which is not ecofriendly with the environment.

The process achieved through the development and selection of superior legume varieties, improves agricultural production particularly in agronomic practice, and increases efficiency of nitrogen-fixing process via better management of the symbiotic relationship between plants and bacteria. The symbioses association between *Rhizobium* and legumes are a cheaper and usually effective agronomic practice for ensuring an adequate supply of nitrogen for legume crop production than application of inorganic nitrogen fertilizers.

3.5. The Macrosymbiont: The Legumes

Legumes are second only to cereals as a source of human food and animal feed. Their importance as food lies primarily in their high protein content, fat and carbohydrates. They are also essential for good health due to its contents in high bone building minerals and vitamins (Porres *et al*., 2003). Some legume crops such as; faba bean, common bean, chickpea, lentils and field pea combined account for about two-thirds offield crop areas covered in Ethiopia. These leguminous crops are known for their ability to form endosymbiotic relationship with the root nodule bacteria collectively called Rhizobia. Faba bean like other legumes crops has the ability

to form symbiotic association with root nodulating bacteria (rhizobia) group called *Rhizobium leguminosarum bv. Vicia*.

3.5.1. Domestication and distribution of faba bean (*Vicia faba*)

Faba bean (*Vicia faba L*.) is a major grain legume crop, grown for high protein content and superior biomass, ranking as the fourth most internationally important cool-season legume after peas, chickpeas and lentils (FAOSTAT, 2014). It is considered to be one of the nine founder crops (or primary domesticates), is probably native to southwestern Asia (Tanno & Willcox, 2006; Zeder, 2009). After its domestication at least 7000 years ago, faba bean spread to Africa, the Mediterranean basin and central Europe, the Americas, China, India, etc. (Ye *et al*., 2003; Duc *et al*., 2010). Ethiopia is one of the largest faba bean producing countries in the world only second to China (Biruk, 2009).

Historically, Ethiopia is considered as the secondary center of diversity and also one of the nine major agro-geographical production regions of faba bean (Asfaw*et al*., 1994). It was grown as field crop throughout the highlands and is most common in Wayena Dega between the altitudes 1800 and 3000 meter elevations (ICARDA, 2006; Temesgen and Aemiro, 2012), with greatest concentration Gondar, Wollo, Gojjam, Shewa, and Tigray regions. According to Crépona *et al*. 2010 the nutrient value of faba bean is high and it has been considered as a meat extender or substitute due to its high protein content (approximately, 20-41 %). It has been produced for centuries in Ethiopia, and used as a daily food for protein supplement to the diet of rural household and source of cash crop to the farmers and foreign currency in the country (ICARDA, 2006).

3.5.2. Nodulation genes and nod factors

A common genetic determinant for rhizobia is the presence of genes encoding nodulation and nitrogen fixation functions (*nod, nol, noe, nif* and *fix* genes). The *nod, nol* and *noe* gene products are involved in production of a nodulation signal, the Nod factor, which is a lipo-chitooligosaccharide. The molecular dialogue between the host plants and the bacteria is an important part for the formation of nodule (Dénarié *et al*, 1993; Schultze and Kondorosi, 1998).

Flavonoids and other secondary metabolites produced by host plants in the rhizosphere induce rhizobial nod genes. This leads to production of *Nod* factors. Signal molecules can be perceived by a specific bacterial receptor, *NodD*, which acts as a transcriptional activator of other nodulation genes. According to Yang *et al*.,(1999) core of the Nod factor molecule is encoded by canonical *nodA,nodB* and *nodC* genes, on the other hand, *nodFE* are involved in polyunsaturation of the fatty acyl group attached to the core molecule. The Nod factor from the bacteria induces root nodule formation in the plant that leading to construction of the root nodule and entry of rhizobia into the nodule (Long, 2001; Gage, 2004).

3.5.3. Nodule physiology

During nodule formation, host tissues develop to form a specialized tissue that maintains an environment in which nitrogen fixation can occur. In the nodule, specialized organelle-like forms of bacteria called bacteroids are engulfed in plant-derived membranes, forming symbiosomes. The reduction in dinitrogen inside the nodule requires energy, which is provided by the plant. Photosynthetic in the form of sucrose is transported to the nodule, whereas dicarboxylic acids further provide the bacteroids with carbon and energy through the symbiosome membrane. For generation of energy through respiration, a high flux but a low internal concentration of oxygen is achieved with the aid of leghemoglobin. Ammonia produced in the bacteroid needs to be transported to the plant through the symbiosome membrane.

3.6. The Microsymbiont: *Rhizobium*

3.6.1. Taxonomy

Symbiotic nitrogen fixing bacteria are represented by a phylogenetically different class of *alpha-* and *beta-proteobacteria*, collectively known as*rhizobia*, which can achieve the function of fixation of atmospheric dinitrogen in symbiotic association with legumes (Michael *et al*, 2012). Michael *et al*., (2012) found that, the majority of the symbiotic species are represented in the *alpha-proteobacteria* order *Rhizobiales*, which contains the agriculturally important nitrogen fixing genera of *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium*. *Rhizobium* species are a soil habitat, rod shaped, non-spore forming and gram-negative bacterium, which can able to colonize the legume roots and fixes atmospheric dinitrogen into

ammonia symbiotically for the benefit of plants. The *rhizobium*–legume symbioses vary in specificity forboth the breadth of host range and the diversity of bacterialspecies nodulating a given host plant. The interactionbetween the tribe *Viciae* (Vicia, Pisum, Lens and Lathyrus)and*Rhizobium leguminosarum*bv. *Viciae* was thoughtto be one of the most specific.

3.6.2. Cultural characteristics of Rhizobium

The growth of most fast-growing rhizobia after 3-5 days consists of extracellular gum and, in some cases, flow over the larger part of the agar surface. The growth of *R. leguminosarum, R. trifolii* and *R. phaseoli* is often clear as water or has differentiated into more opaque areas set in clear surroundings. Some are evenly mucoid throughout. Growth of *R. meliloti* is often more evenly opaque, even chalky white rather than clear or milky. Colonies of slow growing strains do not become apparent for several days; they require about 10 days to reach maximum size and do not produce free-flowing gum.

The ingredient of Congo red dye has been generally used as a differential media for rhizobia (Vincent, 1970). Generally, rhizobia do not absorb the dye but the others do. Similarly, the addition of Bromothymol-blue provides a useful indicator of relatively small changes in acidity or alkalinity. Yeast extract mannitol medium, generally known as medium 79 (Allen, 1959), is suitable for growth of rhizobia. Most strains of rhizobia grow, poorly on glucose peptone agar but some, *R. meliloti* and *Agrobacterium*, make a reasonable degree of growth on this medium.

Growth of rhizobia in liquid media is generally evenly turbid and may become extremely viscous due to the production of gum. Reactions in litmus milk have often been taken to be distinctive between *R. meliloti* (acid reaction) and the slightly alkaline reaction found with other species. The production of a serum zone appears as a strain specific rather than a species characteristic.

3.6.3. Physiology of *Rhizobium*

The Physiology of *Rhizobium* described or reported in (Bergesen, 1978). A few points of practical significance are mentioned here.

Carbon source: In general, *R. leguminosarum*, *R. trifolii*, *R. phaseoli* and *R. meliloti* make good use of all carbon sources. The slow growers fail to use rhamnose, sucrose, trehalose, raffinose and dulcitol. Maltose, lactose and organic acids vary a good deal between strains.

Nitrogen source: Many strains can utilize nitrate or ammonium as the sole or supplementary source of nitrogen but care should be taken to avoid a change in pH. Strains unable to use inorganic combined nitrogen are generally satisfied by the addition of a single amino acid. In strains that differ in their ability to utilize certain amino acids, glutamic acid is generally acceptable. About 5 - 10 ppm nitrogen are required for optimum growth of *rhizobia*; multiplication and respiration rates are augmented in media containing 108 ppm. Urea is utilized by a large collection of representative strains and species, and most show a moderate degree of growth on biuret.

pH: In general all Rhizobia grow within a pH range of 5.5-7.5.

Temperature: From available data, *Rhizobium* grows over the range 0-50°C with the optimum near 20 - 28°C. Among temperate strains, those of *R. meliloti* are the most tolerant (36.5-42.5°C), being 8°C higher on the average than those of *R. leguminosarum* and *R. trifolii* (31-38°C).

3.7. Methods of assessing Biological Nitrogen-fixation

The assessment of the nitrogen-fixing ability is vital for direct determination of total nitrogen gains in or indirectly the nitrogenase activity. Some important techniques of assessing Nitrogen-fixation are described below:

3.7.1. Kjeldahl Method

An analytical method for the determination of nitrogen content was introduced in 1883, by Kjeldahl, a Danish scientist and was the only available method until the 1940's (Bergersen, 1980). It is useful for assessing the response to inoculation, especially in laboratories lacking the specialized equipment required for other methods, although it cannot be used to present conclusive evidence of the capacity of an organism to fix nitrogen. Details on equipment needed,

procedure and calculations are presented by (Allen, 1959) and (Vincent, 1970).The Kjeldahl technique has a paramount important to quantify nitrogen in different ecosystems.

3.7.2. ^{15}N Method

After Kjeldahl method, ^{15}N isotopic techniques (in the 1940's) used by scientists to demonstrate a gain in nitrogen resulted from nitrogen fixation more effectively (Burris and Miller, 1941). Based on the Boddey *et al*; 2000 and 2001 the use of ^{15}N isotopic gas is impracticable in field conditions. Instead, a common method is based on differences in the natural abundance of ^{15}N contained in mineral sources of nitrogen compared to atmospheric nitrogen. It enables determining the proportion of plant N derived from atmospheric nitrogen (%Ndfa). The method provides a great understanding of biochemistry and bacteriology of nitrogen-fixation during the 1940's and 1950's and it is 1000-fold more sensitive than the Kjeldahl method (Claudine *et al*., 2009).

3.7.3. Acetylene Reduction

An assay using the acetylene reduction technique, which is a major achievement for rapid determination of nitrogenase activity (Hardy *et al.*, 1973) and enable to apply on pure cultures and cellular extracts, greenhouse experiments in legumes and non-legumes, to excised roots, and soil cores (Bergersen, 1980). In this assay method, nitrogenase can reduce alternative substrates such as acetylene into ethylene, determined by gas chromatography (Dilworth, 1966; Claudine *et al*., 2009). Essentially, the method involves incubating the test material in a gas tight container which contains partial pressure of acetylene. At appropriate times samples of the atmosphere may be withdrawn by syringe for immediate or eventual analysis by using flame ionization after gas chromatography. The amount of C_2H_4 detected is correlated with the intensity of nitrogenase activity in the sample. Values for nitrogenase obtained by this method are conventionally designated as N_2 (C_2H_2) fixing activity and nitrogenase (C_2H_2) activity. According to Burns and Hardy, (1975) the method is about 10^3 times more sensitive and relatively inexpensive for use than $^{15}N_2$.

3.8. Effects of severe conditions on nitrogen fixation

There are several environmental conditions which can limit the growth and activity of the N_2-fixing system in plants. In *Rhizobium*-legume association, N_2 fixation is strongly related to the physiological state of the host plant. Photosynthetic deprivation, water stress, salinity, soil nitrate, temperature, and heavy metals are limiting factors from the environment that can reduce the symbiotic association between *rhizobium* and the legumes to be effective (Walsh, 1995). These environmental stress has more than one impact, for instance, salinity may act as a water stress, which affects the photosynthetic rate, or nodule metabolism directly. Bottomley, 1991 found that marginal lands with low rainfall, extremes of temperature, acidic soils of low nutrient status, and poor water- holding capacity of soil are the most determinant factor from the environments for *rhizobium* activities. Biological nitrogen fixation enables to improve agricultural productivity while minimizing soil loss and ameliorating adverse edaphic conditions are essential.

3.8.1. Salt and osmotic stresses

Salinity is a serious threat for effective agriculture production. Increasing salt concentration in the soils or water has a detrimental effect on productivity and growth patterns of most crop plants (Cordovilla *et al.,* 1994) and decrease microbial populations as a result of direct toxicity through osmotic stress (Tate, 1995). Depending upon the plant species, salinity levels, and ionic composition of the salts the plant growth and yield can be decreased (Delgado *et al*., 1994). In most cultivated crops, the legumes, the salinity response varies greatly and relay on climatic conditions, soil properties, and growth stage of the plant (Cordovilla *et al*., 1995). Reports from (Zahran, 1991), there is variability in salt tolerance among legume crops. For instance, *Vicia faba*, *Phaseolus vulgaris* and *Glycine max*, are more salt tolerant than *Pisum sativum*. Some studies show that, *Vicia faba* tolerant lines preferable for nitrogen fixation under saline conditions (Abdel-Wahab and Zahran, 1981; Cordovilla *et al.,* 1995). The association between legume-*Rhizobium* symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the *rhizobium* itself (Zahran and Sprent, 1986; El-Shinnawi *et al*., 1989; Velagaleti *et al*., 1990; Zahran, 1991). Above optimum polyethylene glycol and NaCl concentration, the *rhizobium* colonization and root hair curling of *V. faba* decreased (Zahran,

1986; Zahran and Sprent, 1986). The reduction of respiration in nodules and protein production, particularly, leghemoglobin, by nodules is the result of reduction in N_2-fixing activity by salt stress (Ikeda *et al*., 1992; Delgado, 1993; Delgado *et al*., 1994; Walsh, 1995). Salt stress has a direct effect on N2 fixation rate, as a result, nitrogen content in the shoot, nodule structure, and photosynthetic activity of legumes reduced (Zahran, 1986; Georgiev and Atkias, 1993; Cordovilla *et al*., 1995; Zahran and Abu-Gharbia, 1995).

Many species of bacteria have osmolytes for adaptation of saline conditions to hinder the dehydration effect of low water activity in the medium but not to interfere with macromolecular structure or function (Csonka and Hanson, 1991; Smith *et al*., 1994). Osmolytes are intracellular accumulation of low-molecular-weight organic solutes utilized by Rhizobium for mechanism of osmotic adaptation (Botsford, 1990; Botsford and Lewis, 1990; Smith *et al*., 1994; Zahran *et al*., 1997). Trehalose is a disaccharide carbohydrate plays a role in osmoregulation when rhizobia are growing under salt or osmotic stress (El-Sheikh and Wood, 1990; Hoelzle and Streeter, 1990). Intracellular accumulation of glycine betaine in rhizobium is vital for identification of salt or osmotic stress response (Sauvage *et al*., 1983; LeRudulier and Bernard, 1986; Smith *et al*., 1988). Glycine betaine is an osmoprotective substance which has a significant role in the maintaining nitrogenase activity in bacteroids and as energy source in family of Rhizobiaceae under salt stress. Chien *et al*. (1992) examined that mutant strains of *R. leguminosarum bv. Viciae* grow at 200 mM NaCl, but the nodules failed to express nitrogenase activity due to ineffective nodules formation on roots of *V. faba*. Though, some strains of *Rhizobium* tolerated extremely high levels of salt (up to 1.88 M NaCl) their symbiotic efficiency under salt stress significantly decreased (Nair *et al*., 1993). Craig *et al*., (1991) showed that tolerance of the legume host to salt is the most important factor in determining the success of compatible *Rhizobium* strains to form successful symbiosis under conditions of high soil salinity.

3.8.2. Soil moisture deficiency

Morphological change in *Rhizobium* is the immediate responses due to water stress. The reduction of infection and nodulation of legumes is the result of soil moisture deficiency in most rhizobium cells. Deprived of water simulated with polyethylene glycol, significantly reduced the formation infection thread and nodulation of faba bean (*Vicia faba*) plant (Zahran, 1986; Zahran

and Sprent, 1986). Not only a favorable rhizosphere environment, but also the magnitude of stress effects and the rate of inhibition of the symbiosis usually phase of growth and development, as well as the severity of the stress is vital to legume-*Rhizobium* interaction. For example, according to (Williams and De Mallorca, 1984), mild water stress reduces only the number of nodules formed on roots of soybean, while moderate and severe water stress reduces both the number and size of nodules.

Soil moisture deficiency has a determinant effect on N_2 fixation since nodule initiation activity and growth are more sensitive to water stress than root and shoot metabolism (Albrecht *et al*., 1994; Zahran and Sprent, 1986). Recently, Sellstedt *et al*. (1993) examined the amount of N from N_2 fixation under acetylene reduction assay technique was decreased by about 26% due to water deficiency. The physiological response mechanisms of several legumes to water stress have been suggested. For instance, accumulation of specific organic solutes (osmolytes) is a characteristic response of plants subject to prolonged severe water stress.

3.8.3. High temperature stress

A major problem for biological nitrogen fixation of legume crops in tropical and subtropical region is high soil temperatures (Michiels *et al*., 1994). High temperature strongly affects root hair infection, bacteroid differentiation, nodule structure, and the functioning of the legume root nodule for N_2 fixation (Roughley, 1970; Roughley and Dart, 1970). Critical temperatures for N_2 fixation are 30°C for clover and pea and range between 35 and 40°C for soybean, guar, peanut, and cowpea (Michiels *et al*., 1994). Strain and the plant cultivar play a significant role for nodulation formation and symbiotic nitrogen fixation (Munevar and Wollum, 1982; Arayankoon *et al*., 1990). Graham, (1992) showed that the optimum temperature range for growth of most rhizobium in culture is 28 to 31°C, and many are unable to grow at 37°C.

3.8.4. Soil acidity and alkalinity

Soil acidity has detrimental effect on agricultural production in many regions of the world and limits yields of legume crops (Clarke *et al*; 1993; Bordeleau and Prevost, 1994). It has been reported (Brockwell *et al*; 1991; Bordeleau and Prevost, 1994) that symbiotic nitrogen fixation in leguminous plant requires a neutral or slightly acidic soil for their growth. Legumes and their

rhizobia exhibit varied responses to acidity. In both tropical and temperate areas, soil acidity is the main problem in symbiotic N2 fixation (Munns, 1986) and a significance constraint in *Rhizobium* survival and persistence in soils and reducing nodulation (Graham *et al*; 1982; Brockwell *et al*; 1991). According to Graham *et al*.,(1982) report, Rhizobia with a higher tolerance to acidity have been identified and Graham *et al*; (1994), these strains usually but not always perform better under acidic soil conditions in the field. In certain cases, pH tolerance varies widely strains of a given species. Generally, fast-growing strains of *rhizobia* have been considered as less tolerant to acid pH than slowly growing strains of *Bradyrhizobium* (Graham *et al*; 1994). Under acid-soil conditions, failure of nodule in legumes is common, particularly, soils of pH less than 5.0. Inoculation with strains of *R. leguminosarum bv. Viciae* in acid soils is effective for the growth, nodulation, and yield of *V. faba* (Carter *et al*; 1994). Vassileva *et al*.,(1997)reveals that, the number of nodules, nitrogenase activity, nodule ultrastructure, and fresh and dry weights of nodules were affected to a greater extent at a low medium pH (4.5). Selecting tolerant plants and liming the acidic soil to ameliorate the effects of acidic conditions are strategies have been adopted to solve the problem of soil acidity.

3.8.5. Nutrient deficiency stress

Nutrient deficiency, mineral toxicity and nutrient disorders are usually outcomes of soil salinity and acidity. Nutrient deficiency of ions of Na^{+1} and Cl_2 might occur and might be balanced by increasing the concentration of counter ions, like K^{+1} and Ca^{+2}, against Cl_2 (Glass, 1983). Na and Cl_2 are the dominant ions in water and soil salinity. Plenty of Na^{+1} in the soil often devastate effect on nonhalophytes by displacing $Ca^{+2}$from root membranes and change their integrity and their normal function (Cramer *et al*., 1985). Acidic stress also affects ion absorption and growth of roots (Torimitsu *et al.,* 1985); as a result the membrane structure and function of the roots suffer fatal changes under these stress conditions. Under severe stress conditions, Ca^{+2} and P are essential elements for plant growth and development. The demand of Ca^{+2} for growth of *R. meliloti* was increased under osmotic stress (Busse and Bottomley, 1989) and deprived of Ca^{+2} in *R. leguminosarum* cell suffer from swollen, lack rigidity, and express an additional somatic antigen normally blocked by side chains of the LPS O antigen (De Maagd *et al*., 1989).

In *Rhizobium* cells, high levels of salinity (i.e. up to 10% NaCl) reduces the content of Ca^{+2} (Zahran *et al*., 1997), and distort their outer membrane structure. Graham, (1992); Torimitsu *et al*.,(1985)founds that values of calcium has vital role in cells exposed to low pH to improve both growth and ion uptake by roots, and Beck and Munns, (1984) shows that phosphorus starved cells or cells grown at low pH needed Ca^{+2} for phosphorus mobilization in the cell. Ca^{+2} controls K^{+1} permeability and activation of K^{+1} uptake through the acidification of the cytoplasm during k transport and plays a key role in cell division, elongation, and membrane structure and function (Torimitsu *et al*., 1985). Zahran and Sprent, (1986) reported that salt stress (100 mM NaCl) decreases the attachment to and colonization of root hairs of *V. faba* plants by *R. leguminosarumbv.viciae*. Salt and acidity stresses have a detriment effect on calcium availability and on the initial stages of nodule formation which will affect the net nodulating capacity of legumes. Phosphorus supply also plays a significant role for both nodulation and N2 fixation (Ssali and Keya, 1983; Pereira and Bliss, 1989).

3.9. Genes and signal molecules involved in the Legume-Rhizobia symbiosis

Symbiotic association of *Rhizobium*species and legumes play a significance role in atmospheric nitrogenfixation for a renewable source of nitrogen for agriculture. This biological process can be appeared under low level of available nitrogen with help of many different genes such as *nod, nif, fix,* production of polysaccharides, infection process, hostspecificity, signals of host and many other different genes involved between *Rhizobium* strains and their legume partner (Abdelaal, 2013). The coordinate interactionof the *nif* genes and *fix* genes found in *rhizobium* are vital for environmental symbiotic nitrogen fixation.There are many different types of *nod* genes designated as *nodA*, *nodB* and *nodC,* which are collectively responsible for the biosynthesis of chitin backbone; on the other hand,*nodD* is a regulatory gene that activates the transcription of other inducible *nod* genes (Martinez*et al*.,1990).

Postgate, 1982 examined that fixation and reduction of atmospheric dinitrogen into ammonia is a sophisticated process which requires a huge amount of energy (ATP). Since the molecule consists of two nitrogen atoms joined by a triple covalent bond which becomes chemically inert and nonreactive (Wagner, 2012).The primary enzyme encoded by *nif* genes is nitrogenase complex.Nitrogenaseis the well-known complex metalloenzyme that plays a vital role in the breaking down of the bond in atmospheric dinitrogen.Nitrogenase consists of two component

proteins called as component I or molybdenum-iron protein(Mo Fe) also known as dinitrogenase and component II or an iron-containing protein (Fe)also known as dinitrogenase reductase (Burris, 1991).

The most important elements for *Rhizobium*-legume communication during the first step of nodule formation is nod factors. Abdelaal, 2013 shows that nod factors also known as lipochitoligosaccharides (LCOs) produced and excreted by more than 30 different *nod*, *nol*, *noe* genes and their corresponding proteins from the microsymbionts. Cyclic Glucans are small molecule linked either by β- (1, 2) glycosidic bonds with 17 to 40 units in *Rhizobium* and *Sinorhizobium* or by β - (1, 3) and β- (1, 6) glycosidic bonds in *Bradyrhizobium japonicum. Rhizobia* produce large quantities of cyclic glucans in the endosymbiotic stage and have a main roleto protect against hypoosmotic conditions in *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* (Abdelaal, 2013). Morris*et al*., 1991 found that increasing the solubility of falvonoids and Nod factors are specific function during symbiosis between legume-rhizobium. Lipopolysaccharides are also produced by *rhizobium* which involves in the infection process (Brewin, 1998) and used in the suppression of the host plant defense response (Schonejans *et al*., 1987). Exopolysaccharides are signals secreted by a large numbers of rhizobia that plays a major role in the primary stage of the infection of the host plant and suppression of a defense response by the host plant (Parnisik *et al*., 1994). Exopolysaccharides are essential for the development of fully functioning of root nodules (Mathis*et al*., 2005).

The relation between *Rhizobium* and its symbiotic partner usually start byproducing biochemical signalsfrom both sides. The legume plants releaseda secondary metabolite product termed as falvonids induced by *nod* factor from the micro-symbiont for communication dialogue between them. Perret*et al*., 2000 examined that more than 4,000 different flavonoids have been identified in vascular plants and involves in mediating host specificity in the legume plants. Not only flavonoids but also betaines, aldonic acids, xanthones, simple phenolics and jasmonatessignals produced in legume plantsto inducers *nod* gene in *rhizobia*(Cooper, 2007). Lectins have been found in the tip of root hairs (Diaz *et al*., 1986) and involved in *Rhizobium*-symbiotic interaction in two ways, first lectins have important function in the infection process and second in the root nodule maturation.

4. MATERIAL AND METHODS

4.1. Study Area

The study was carried out in University of Gondar, Department of Biotechnology at Molecular Biology laboratory from January, 2016 to November, 2016.

4.2. Study design

The study design was cross-sectional using appropriate methods such as authentication of isolates test, biochemical characterization, physiological characterization and morphological characterization activities were performed from January, 2016 to November, 2016. The experiment was laid on completely randomized design (CRD) design with three replications for each treatment and control.

4.3. Collection and Identification of Soil sampling

Three representative faba bean growing woredas were selected from North Gondar and South Gondar zone (Laygayint and Farta woreda from South Gondar; Chilga from North Gondar) based on their productivity of faba bean in the last few years. From each woreda, the productive areas were selected based on their productivity of faba bean. From each woredas of productive area, representative farmer fields were selected based on the previous history of cultivating faba bean crop. Thus, twenty farmer fields from Chilga, twenty farmer fields from Farta and seventeen farmer fields from Lay Gayint were selected as shown on (Fig. 1). About 3 Kgs of soil sample was collected from each selected farmer fields at a depth of 20 cm using sterile (fresh) plastic bags. The soil samples were transported to the Department of Biotechnology, Molecular Biology laboratory room in University of Gondar for further work. The soil samples were characterized for their texture, pH, phosphorus content (ppm), nitrogen content (ppm) and potassiumcontent (ppm). Available Phosphorus (ppm) in the soil determined based on Olsen method soil tests as, low (<10), medium (10-20), high (20-40) and excessive (>40) (Olsen *et a1*., 1954). Total nitrogen (%) of the soil was determined by Kje1dahl technique as outlined by (Tekalign, 1991) and the interpretation was classified as very low (<0.05), low (0.05-0.12), moderate (0.12-0.25) and high (>0.25).Potassium (ppm) availability in the soil determined based

on ammonium acetate method by Pratt, (1965) and the interpretation was in the following way: very low (< 75), low (75-150), medium (150-250), high (250-800) and very high (> 800).

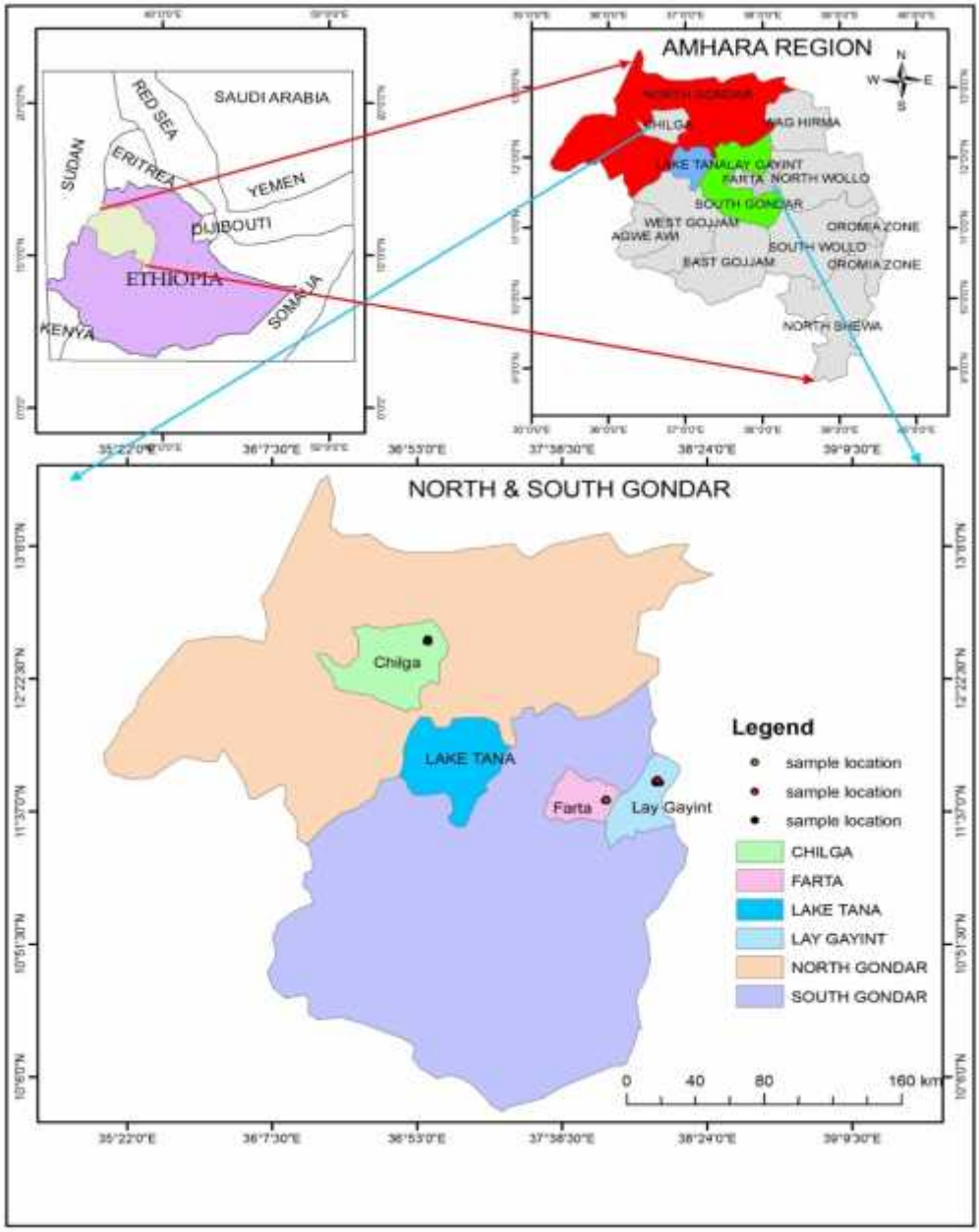


Figure 1: Map of the Soil sampling location in North and South Gondar Zones in Amhara Region.

4.4. Induction of Nodulation (Presumptive test)

Rhizobium was isolated from the soil samples collected from each sites by plant infection method (Vincent, 1970; Somasegarian and Hoben, 1994). The soil samples were placed in a 3kg capacity

plastic pots that were surface sterilized with 70% ethyl alcohol. Healthy faba bean seeds were surface sterilized with ethyl alcohol for 10 seconds and then with 3% H_2O_2 for three minutes (Vincent, 1970). After rinsing five times with sterile water, five seeds were planted on each pot, which were further thinned down to three. The pots were placed in green house and watered until field capacity with sterile water twice a week for 45 days. Nodule number, nodule fresh weights, nodule color, shoots height and leaf colors (yellow, green) were determined from each pot.

4.5. Isolation of root nodule bacteria

Rhizobium was isolated from nodules as described by Vincent (1970). Three healthy nodules were randomly picked from every plant; surface sterilized as before and was crushed with sterile glass rod. Loopful of the crushed materials was streaked on to plates of Yeast Extract Mannitol agar (YEMA) medium (Vincent, 1970) and was incubated at 30^{o}C for three to five days. The components of YEMA were as reported earlier(Somasegaran and Hoben, 1994); which contained; 10g/l Mannitol, 3mM K_2HPO_4, 0.8mM $MgSO_4.7H_2O$, 2mMNaCl, 1g/l Yeast Extract, and 20g/l Agar. After incubation, single colony was subcultured several times and preserved on YEMA slants containing 0.3% (w/v) $CaCO_3$ at 4 ^{o}C.

4.6. Morphological Characterization

Colony Morphology of isolates was examined on YEM agar plates. Approximately 10^9 cells per ml culture were spread on YEM agar. After incubation for 3-5 days at 30 ^{o}C, individual colony was characterized on the basis of colony- shape, size, and color (Jordan, 1984).

4.7. Biochemical characterization

4.7.1. Acid or Alkali production

The production of acid or alkali (i.e. whether the isolates are fast growing or slow growing) were evaluated onYEMA containing 25 µg ml^{-1} bromothymol blue (BTB) (Lupwayi and Haque, 1994) as reaction indicator and were shaked by orbital shaker.

4.7.2. Growth on Congo red-YEMA

The coexistence of contaminants were checked via three days old yeast extract mannitol broth cultures that were streaked on YEMA media containing 0.0025% (w/v) final concentration of Congo red (YEMA-CR).

4.7.3. Gram staining

Gram staining was conducted for all isolates as a means for rapid identification of gram-positive contaminants as the method indicated byLupwayi and Haque (1994).

4.8. Physiological characterization

4.8.1. Utilization of Carbon source

All the isolates were tested on fifteen carbohydrates as a sole source of carbon (Glucose, Fructose, Xylose, Sucrose, Lactose, Adonitol, Inositol, Sorbitol, Raffinose, Arabinose, Mannose, Glycerol, Dextrose, Rhamnose, and Maltose). Stock solutions of each carbohydrate were prepared at 10% (w/v) in sterile distilled water (Somasegarian and Hoben, 1994). Each 10 ml of the carbohydrate stock solutions were added to 90 ml of the carbohydrate free basal medium and their growth were observed after 3-5 days.

4.8.2. pH tolerance

Cells were allowed to grow in 10ml YEMB. The capacity of each rhizobial isolates to grow on acidic and alkaline media were determined by inoculating a loopful of each isolate on YEMA adjusted at a pH of 4.0, 4.5, 5.0, 5.5, 6, 8.0, and 9.0 using sterile 1N HCl and NaOH before autoclaving and incubated at 30^{o}C for 3- 5 days (McVicar *et al*., 2005).

4.8.3. Salt tolerance

Each isolate was allowed to grow in 10 ml YEMB and loopful of each inoculum was inoculated on petridishes containing YEMA adjusted to 0.1, 0.5, 1, 2, 3, 4, and 5% (w/v) concentrations of NaCl and incubated at 30^{o}C for 3- 5 days (Amarger *et al*., 1997 and McVicar *et al*., 2005).

4.8.4. Temperature tolerance

The ability of bacterial strains to grow at different temperatures weremonitored by incubating them on solid YEMA medium. Petriplates containing YEMA was inoculated with 10µl of 48hr bacterial cultures and incubated to 5^{o}C, 10^{o}C, 15^{o}C, 35^{o}C, 40^{o}C and 45^{o}C for 3-5 days, according to (Jordan, 1984 and McVicar *et al*., 2005).

4.8.5. Intrinsic antibiotic resistance

Intrinsic antibiotic resistance study was under taken in the laboratory by preparing stoke solutions (10mg/ml) of antibiotics in the appropriate solvent, filter sterilized with Millipore filters and stored at 4 ^{o}C. The antibiotics (in µg) used were at concentrations of: Erythromycin (100, 125, 150, 200), Ampicillin (100, 125, 150, 200), Tetracycline (5, 10, 15, 25) and Chloramphenicol (5, 10, 15, 25). Stock solution of Erythromycin and Tetracycline were dissolved in50 % ethanol where as Chloramphenicol and Ampicillinweredissolved in sterile distilled water, according to Taye (2010). Each of filter sterilized antibiotics were added aseptically in appropriate amounts of sterile YEMA medium that had been cooled and were mixed thoroughly. Then twenty milliliter of the medium was poured into sterile petriplates and after cooling 10 µl of each culture (10^{9}cells/ml) was inoculated and incubated for 5 days at 30^{o}C.Result for all physiological growth tests were determined qualitatively and presented as '+' for growth and '-'for no growth.

4.9. Authentication of the strains (Symbiotic effectiveness)

In order to determine the isolates performance (effectiveness) in nitrogen fixation, authentication test was done as described by Vincent (1970). Out of 57 isolates, 40 best isolates were selected from the presumptive test based on nodule color, nodule number, nodule fresh weight and shoot height. For each isolate three surface sterilized pots were filled with approximately 3kg acid washed and heat sterilized river sand. In each pot four to five healthy surface sterilized Adet faba bean variety seeds from Adet Agricultural Research Centre were germinated and planted according to the methods of Somasegarian and Hoben(1994). A total of 120 pots were required for this experiment. As a starter, 20 ppm of nitrogen was included in each pot before planting (Gibson, 1980).Each seedling was inoculated with 1 mlof each isolate with an inoculum size of 10^{9}cells/ml (Somasegaran and Hoben, 1994).After a week, the seedlings were reduced into three

per pot. Two treatments were used as control: one without nitrogen supply and an uninoculated (i.e. negative control) and the other an uninoculated but with provision of 0.05% (W/V) KNO_3per week (i.e. positive control). This experiment was made in triplicates and the plants were grown under glasshouse condition. The pots were arranged in Completely Randomized Design (CRD) and plants were also fertilized with the quarter strength Broughton and Dilworth nitrogen-free nutrient solution once a week and received water every three days (Broughton and Dilworth 1970), cited in (Somasegaran and Hoben, 1994). After 45 days of planting, the plants were carefully uprooted and nodule color, nodule number, nodule fresh weight, shoot length were counted and measured, nodule dry weight, root biomass and shoot dry weight were also measured after drying at 70 ºC for 24 hr under dry oven, and total nitrogen were analyzedby kjeldhal method (Lupwayi and Haque, 1994).

The Relative symbiotic effectiveness of the isolates were calculated according to the equation proposed by Date *et al.* (1993) in Purchino *et al.* (2000) (100 X inoculated plant DM/ N-fertilized plant DM) with Nitrogenfixing effectiveness classified as ineffective<35%; lowly-effective, 35 to 50%; effective, 50 to 80%; and highly effective, >80%.

4.10. Nodulation status survey

The general survey was conducted during the identification and collection of soil samples. In most cases, attempts were made to meet the owner of the field to establish cropping history and the owner of the field told or informed as, their field has been covered with faba bean crop.

4.11. Data analysis

Statistical data analysis was done by using SAS software version 9.2. Analysis of variance (ANOVA) was done for the comparison of means for all treatments and Duncan's multiple range test were used to detect the significant difference among treatment means at $p \leq 0.05$.Correlation coefficients were calculated to study the association among the measurement traits using Pearson correlations. Data from all physiological studies were also used for cluster analysis and similarities between isolates and a dendrogram was constructed based on average linkage hierarchical cluster analysis between groups using SPSS version 20.0 software statistical program**.**

5. RESULTS

5.1. Characterization of Soil Samples

The soil samples collected from Chilga (20), Kimir Dingay (20) and Lay Gayint (17) were analyzed at Adet Agricultural Research Center for their texture, pH, phosphorus content (ppm), nitrogen content (%) and potassium content (ppm)used in this study (Table 1). All the soil samples were collected from the altitude ranging from 2001masl to 3182 masl as shown on (Table 1).

The results of soil texture analysis (Table 1) showed that Chilga soils were characterized with 80%,15% and 5 % medium loam, clay loam and sandy loam, respectively. The pH of these soils was recorded ranging from 6.32 to7.34; nitrogen availability was recorded ranging from 0.021-0.203%; phosphorus availability was recorded ranging from 4.935-34.840 ppmand potassium availability was recorded ranging from 180.1402- 531.2617ppm.On the other hand, soils collected from Kimir Dingay area were with 50%, 25%, 20% and 5% clay loam, clay, silty clay loam and silt loam respectively for soil texture. The pH of this soil was recorded rangingfrom 5.28 to 7.4; nitrogen availability was recorded ranging from 0.114 to 0.318 %; phosphorus availability was recorded ranging from 2.447 to 56.235ppm and potassium availability was recorded ranging from 106.168 to 566.869ppm. Whereas, soils collected from Lay Gayint study area were also characterized with 59%, 29%,6% and 6% medium loam, silt loam, clay and silt clay loam soil texture class name, respectively. The pH of these soils was recorded ranging from 5.33 to 6.83; availability of nitrogen was recorded ranging from between 0.035 to 0.312%; phosphorus availability was recorded ranging from 6.064 to 39.096ppm and potassium availability was recorded ranging from 91.12 to 507.85ppm (Table 1).

The results of this finding revealed that, the soil texture from Chilga weremedium loam with 80%, half of soil samples (50%) from Kimir Dingay were categorized as clay loam and from Lay Gayint 59%of the soil samples were grouped to medium loam soil texture.The pH of the soil from Chilga, Kimir Dingay and Lay Gayint were neutral, moderate acidity to neutral and acidic, respectively.The total soil nitrogen value from Chilga study sites was 65%, 30% and 5% with soil samples of moderate, low and very low, respectively. Whereas, 90%, 5% and 5% of soil samples collected from Kimir Dingay were categorized moderate, high and low, respectively and

85%, 5%, 5% and 5% of soil samples collected from Lay Gayint also showed moderate, high, low and very low, respectively. The result of this technique showed that the available nitrogen in all study samples had moderate nitrogen (Table 1).

Phosphorus availability from soil samples of Chilga study area were 50%, 30% and 20% categorized as low, medium and high, respectively. On the other hand., 50%, 25%, 10% and 15% of soil samples collected from Kimir Dingay study area were grouped to low, medium, high and excessive amount of phosphorus respectively.Similarly 29.4%, 41.2% and 29.4% of soil samples from Lay Gayintshowed low, medium and high phosphorus, respectively. In Kimir Dingay area the available phosphorus in the soil samples was 40 ppm and 20 – 40ppm (Table 1).

Eighty five percent (85%) of soil samples collected from Chilga showed high potassium availabilityand only 15% of soil samples had shown medium potassium availability. Whereas, 5%, 5% and 90% of soil samples collected from Kimir Dingay were categorized as low, medium and high available of potassium respectively. On the other hand, 5.9%, 23.5%, 23.5% and 47.1% soil samples collected from Lay Gayint had very low, low, medium and high potassium availabilityrespectively (Table 1).This result indicated that nearly all soil samples from all study areashad contained high availability of potassium.

Table 1: The soil samples characterized for their texture, pH, phosphorus (ppm), nitrogen (%) and potassium (ppm) used in this study.

Soil site	altitude (m)	Soil texture				Soil pH	N (%)	P(ppm)	K (ppm)
		%clay	%Silt	%Sand	Class name				
CHI-1	2001	24	46	30	Medium loam	6.56	0.159	13.327	462.5701
CHI-2	2006	26	40	34	Medium loam	6.89	0.162	6.058	483.4579
CHI-3	2005	24	32	44	Medium loam	6.94	0.084	5.762	180.1402
CHI-4	2009	10	34	56	Sandy loam	7.01	0.072	6.708	227.6168
CHI-5	2016	16	36	48	Medium loam	6.32	0.108	34.840	531.2617
CHI-6	2014	18	42	40	Medium loam	7.11	0.105	11.673	395.7944
CHI-7	2019	20	30	50	Medium loam	6.84	0.138	4.935	270.7477
CHI-8	2018	28	36	36	Clay loam	6.87	0.142	28.753	441.4486
CHI-9	2021	18	40	42	Medium loam	7.05	0.195	11.673	368.5047
CHI-10	2020	22	40	38	Medium loam	6.93	0.092	30.644	444.8131
CHI-11	2025	22	38	40	Medium loam	7.28	0.021	7.063	307.1495
CHI-12	2015	26	32	42	Medium loam	7.03	0.148	8.126	524.0187
CHI-13	2006	22	40	38	Medium loam	6.68	0.202	29.462	429.0187
CHI-14	2031	28	34	38	Clay loam	6.99	0.123	8.836	418.7383
CHI-15	2027	26	38	36	Medium loam	7.03	0.203	9.309	400.1869
CHI-16	2022	24	44	32	Medium loam	6.96	0.100	19.710	522.7103
CHI-17	2018	24	36	40	Medium loam	7.34	0.125	12.027	321.9626
CHI-18	2022	20	40	40	Medium loam	6.88	0.174	7.358	186.5888
CHI-19	2024	34	34	32	Clay loam	6.84	0.137	6.590	434.6729
CHI-20	2017	24	38	38	Medium loam	6.94	0.197	15.573	469.0654
KD-1	3118	36	44	20	Silty clay loam	5.76	0.182	23.316	380.0467
KD-2	3173	34	44	22	Clay loam	6.33	0.174	44.060	526.8692
KD-3	3169	38	38	24	Clay loam	6.16	0.225	25.030	526.2617
KD-4	3167	38	42	20	Silty clay loam	5.93	0.227	16.814	482.9439
KD-5	3177	34	44	22	Clay loam	5.56	0.226	16.460	321.5888
KD-6	3182	26	50	24	Silt loam	6.16	0.114	52.807	320.3738
KD-7	3131	36	44	20	Silty clay loam	7.44	0.190	56.235	566.8692
KD-8	3096	30	44	26	Clay loam	6.21	0.179	8.836	350.5607
KD-9	3095	36	42	22	Clay loam	6.09	0.157	6.176	265.514
KD-10	3107	34	36	30	Clay loam	5.97	0.164	10.845	106.1682
KD-11	3127	44	30	26	Clay	6.51	0.184	5.521	389.1589
KD-12	3088	46	30	24	Clay	6.14	0.196	4.135	221.9626
KD-13	3061	30	32	38	Clay loam	5.44	0.318	2.447	280.9813
KD-14	3040	38	40	22	Clay loam	5.88	0.205	6.124	274.2056
KD-15	3030	36	34	30	Clay loam	5.97	0.197	11.308	371.1215
KD-16	3032	50	34	16	Clay	5.67	0.230	5.220	382.5234

Table 1. Continued ……..

KD-17	3038	38	38	24	Clay loam	6.18	0.186	7.752	262.2897
KD-18	3018	40	38	22	Clay	6.48	0.163	5.039	338.1308
KD-19	3031	42	36	22	Clay	6.17	0.240	9.560	341.3084
KD-20	3041	32	48	20	Silty clay loam	5.28	0.229	12.996	450.9813
LG-1	2145	22	52	26	Silt loam	5.74	0.143	11.127	46.49533
LG-2	2145	18	50	32	Silt loam	6.27	0.108	17.637	408.6449
LG-3	2145	24	40	36	Medium loam	6.34	0.193	23.062	419.4393
LG-4	2200	28	52	20	Silty clay loam	5.4	0.227	11.248	427.9439
LG-5	2210	26	44	30	Medium loam	5.33	0.149	12.634	302.4299
LG-6	2220	24	48	28	Medium loam	6	0.184	8.837	190.7009
LG-7	2205	22	48	30	Medium loam	5.7	0.243	11.609	211.5421
LG-8	2225	26	46	28	Medium loam	5.52	0.192	8.656	91.1215
LG-9	2230	44	38	18	Clay	5.82	0.035	6.064	170.4206
LG-10	2215	18	44	38	Medium loam	6.65	0.130	11.971	148.1776
LG-11	2215	26	48	26	Medium loam	5.8	0.151	11.549	110.7009
LG-12	2220	22	52	26	Silt loam	6.1	0.213	9.922	142.3832
LG-13	2215	24	56	20	Silt loam	5.36	0.217	7.269	192.5234
LG-14	2200	26	46	28	Medium loam	6.83	0.222	35.720	455.3271
LG-15	2200	26	50	24	Silt loam	6.02	0.312	39.096	507.8505
LG-16	2220	14	36	50	Medium loam	5.85	0.195	36.986	460.3271
LG-17	2215	18	36	46	Medium loam	6.18	0.156	26.197	389.1589

CHI= Chilga, KD=Kimir Dingay, LG= Lay Gayint, ppm= parts per million, N= nitrogen, P= phosphorus, K= potassium

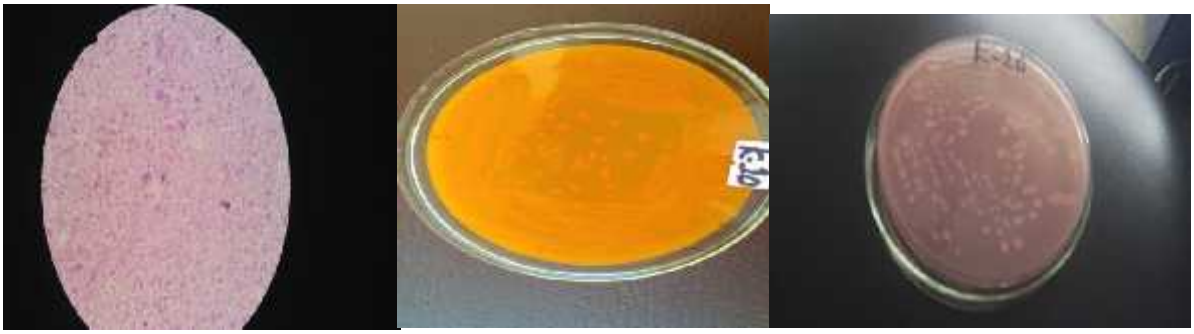
5.2. Induction of Nodulation (Presumptive test)

Rhizobium isolates were obtained from all soil sampling sites. This study showed that variation among faba bean producing areas in terms of nodule number, nodule fresh weight, shoot height and nodule color is presented on (Appendix A: Table 1).The highest average nodule number, fresh weight and shoot height observed from Chilga (CHI-11) was172, 1.05g and 32.5cm, respectively. On the other hand, the highest average nodule number, fresh weight and shoot height fromKimirDingay (KD-4) was 158, 1.08g and 31cm and in Lay Gayint (LG-15) the above traits were 178, 1.08g and 28cm,respectively. The color of nodules of these isolates was pink and the data for induction of nodulation were used for authentication tests or symbiotic effectiveness of isolates on sand culture using pot experiment based on their nodule color, nodule number, nodule fresh weight and shoot height.

Figure 2: Sample of nodulation from Chilga sites taken during induction using pot experiment.

5.3. Morphological and Biochemical characteristics

Fifty seven *Rhizobium* isolates were characterized for their morphological and biochemical tests isolated from Chilga (20), Kimir Dingay (20) and Lay Gayint (17) as shown on (Appendix A: Table 2). Allisolates were gram-negative, rod-shaped; the color of colonies was milky-white opaque with a circular shape, with regular borders and raised, showing intermediate to high production of mucus after 3 to 5 day of growth on YEMA medium at 30 ^{o}C. The isolates changed the YEMA-BTB medium to yellow color and did not absorb Congo red on YEMA-CR media. Furthermore, the colony diameter of all isolates of *Rhizobium leguminosarum*bv.*viciae*was within the range of 1.5 to 4.5mm after 3 to 5 days of incubation at 30 ^{o}C. Isolate CHI-9 from Chilga, LG-7 and LG-16 isolates from Lay Gayint showed the lowest colony size of 1.5mm while, LG-2 and LG-9 isolates from Lay Gayint displayed the highest colony size of 4.5mm(Appendix A: Table 2).



Grams staining Acid production on YEMA-BTB Growth on CR-YEMA

Figure 3: Sample of colony morphology and biochemical tests of *Rhizobium* isolates.

5.4. Physiological characterization of isolates

5.4.1. Utilization of Carbon source

The isolates utilized 100% of Glucose, Fructose, Xylose, Sucrose, Adonitol, Inositol, sorbitol, Raffinose, Glycerol, Dextrose, Rhamnose, and Maltose as sole sources of carbon for their growth (Table 2). With regard to the remaining carbohydrates the rhizobial strains exhibited less diversity in their growth. All strains were able to catabolized Lactose, which accounts for 93% isolates (Table 2) with the exception of CHI-2, CHI-9, CHI-10 and CHI-11 from Chilga (Appendix A: Table 3). Eighty six percent (86%) of isolates had utilized Arabinose as a major sources of carbon for their growth (Table 2) except CHI-8, CHI-11, CHI-15, CHI-18 from Chilga, KD-3 and KD-7 from Kimir Dingay and LG-4 and LG-10 from Lay Gayint (Appendix A: Table 3) and ninety five percent (95%)of isolates had metabolized mannose for their growth (Table 2) except KD-7, KD-14and KD-18 from Kimir Dingay (Appendix A: Table 3). The isolates were found to utilize 86 to 100% of the tested carbohydrates which were served as good sources for the growth of rhizobial (Table 2).

Table 2: Carbon source Utilization by Rhizobium isolates

Carbon sources	**% of isolates growing on the carbohydrate**
Glucose, Fructose, Xylose, Sucrose, Adonitol, Inositol, sorbitol, Raffinose, Glycerol, Dextrose, Rhamnose and Maltose	100
Lactose	93
Arabinose	86
Mannose	95

5.4.2. pH tolerance

Differences in pH tolerance were shown among isolates(Fig.4). Most isolates were grown on a wide range of moderate acidity to alkalinity (5 to 9 pH) and did not grow at the tested pH value of 4 and 4.5 from all soil sample sites (Fig. 4). Fifteen, twelve and ten isolates were grown at the

pH value of 5 from Chilga, Kimir Dingay and Lay Gayint soil sites, respectively. All isolates were grown on the pH range of 5.5 to 8 from all study sites. About 9 isolates for each study site (a total of 27 isolates among 57) were also grown on the pH value of 9 (Fig. 4).

5.4.3. Salt tolerance

All of the 57isolates were tested for their salt tolerance under different concentrations of NaCl using YEMA medium. None of isolates from Chilga, 9 isolates from Kimir Dingay(KD-1, KD-4, KD-7, KD-8, KD-9, KD-13, KD-14, KD-15 and KD-20) and 4 isolates from Lay Gayint soil sites (LG-3, LG-6, LG-8 and LG-12) were grown on the medium containing 0.1% of NaCl (Fig. 5). However, there were 22 (38.6%) isolates grown on the medium containing 0.5 % NaCl out of 57 from all soil sites; 8 isolates (CHI-1, CHI-3, CHI-8, CHI-9, CHI-14, CHI-15, CHI-16 and CHI-20) from Chilga, 7 isolates (KD-2, KD-3, KD-11, KD-12, KD-16, KD-18 and KD-19) from Kimir Dingay and 7 of them (LG-1, LG-2, LG-5, LG-9, LG-13,LG-15 and LG-17) from Lay Gayint soil sites (Fig. 5). On the other hand, isolates grew on the medium containing 1% NaCl were 16 (28%) out of 57 from all soil sites; 9 isolates (CHI-2, CHI-5, CHI-6, CHI-7, CHI-10, CHI-11, CHI-13, CHI-17 and CHI-19) from Chilga, 4 isolates (KD-5, KD-6, KD-10 and KD-17) from Kimir Dingay and 3 of them (LG-4, LG-11 and LG-16) from Lay Gayint soil sites (Fig. 5). Whereas, isolates grew on the medium containing 2% NaCl were also 6 (10.5%) out of 57 from all soil sites; 3 isolates (CHI-4, CHI-12 and CHI-18) from Chilga, no isolates from Kimir Dingay and 3 of them (LG-7, LG-10 and LG-14) from Lay Gayint soil sites (Fig. 5).

5.4.4. Temperature tolerance

All isolates of *Rhizobium* from all soil sites were examined for their growth at different temperatures ranging between 5 – 45 ^{o}C (Fig. 6). All*Rhizobium* isolates showed growthat 15 and 35°C from all study areas. On the other hand, no isolates from Chilga, 14 isolates (KD-1, KD-2, KD-3, KD-4, KD-5, KD-6, KD-7, KD-8, KD-9, KD-10, KD-13, KD-14, KD-17 and KD-20) from Kimir Dingay and 6 isolates (LG-3, LG-4, LG-10, LG-13, LG-14 and LG-16) from Lay Gayint soil sites were grown at 5 ^{o}C. Whereas, there were 43 (75.4%) isolates grown at 10 ^{o}C out of 57 from all soil sites; 6 isolates (CHI-3, CHI-9, CHI-11, CHI-13, CHI-17 and CHI-118) from Chilga, all 20 isolates from Kimir Dingay and all 17 isolates from Lay Gayint soil sites (Fig. 6). There were also 26 (45.6%) isolates grew at temperature of 40 ^{o}C out of 57 from all soil sites; all 20 isolates from Chilga, 4 isolates (KD-3, KD-4, KD-8 and KD-14) from Kimir Dingay and 2 of

them isolates (LG-3 and LG-12) from Lay Gayint soil sites (Fig. 6). On the other hand, 6 (10.5%) isolates were also grown at temperature of 45 ^{o}C from 57 isolates from all study areas; all 6 isolates grown out of 57 isolates (CHI-3, CHI-9, CHI-11, CHI-13, CHI-17 and CHI-118) only from Chilga and none of isolates were grown from Kimir Dingay and Lay Gayint soil site areas (Fig. 6).

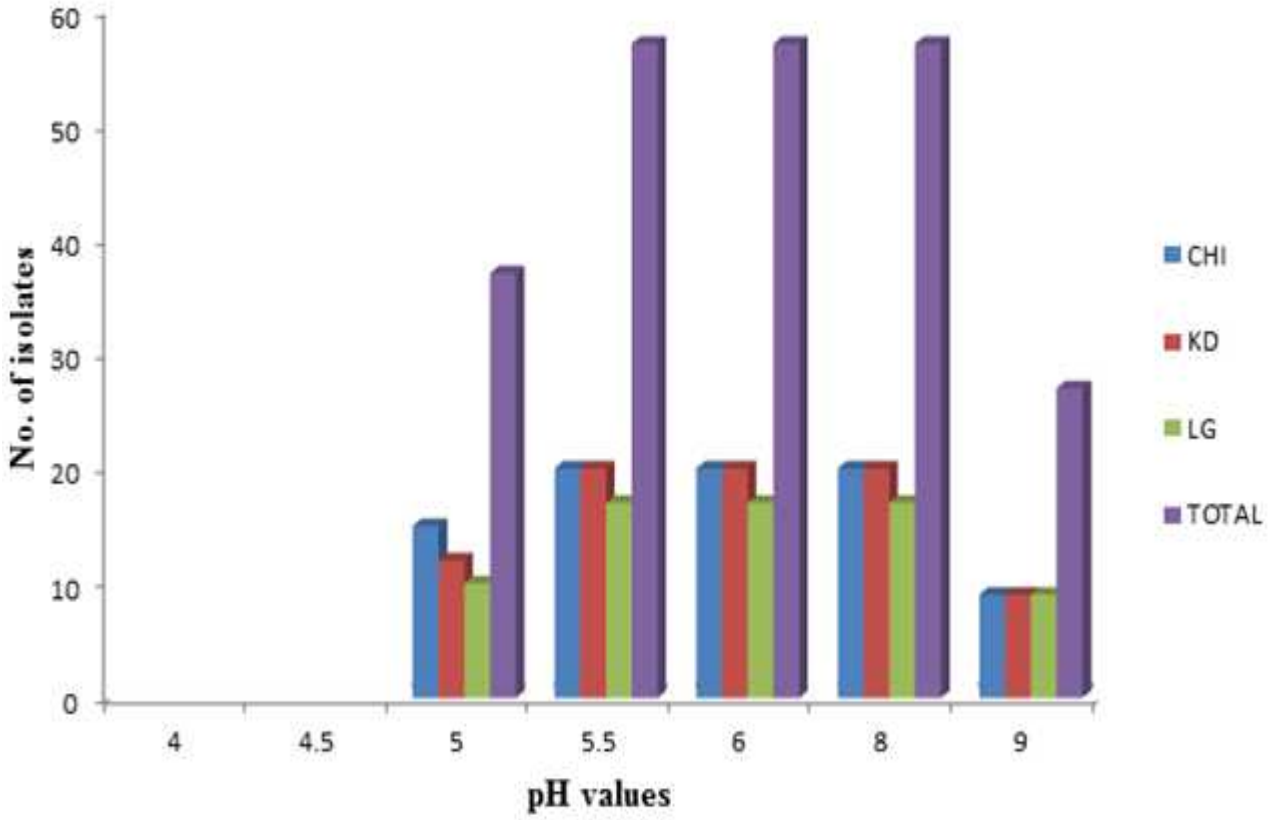


Figure 4: Effect of pH on the growth of *Rhizobium* isolates.

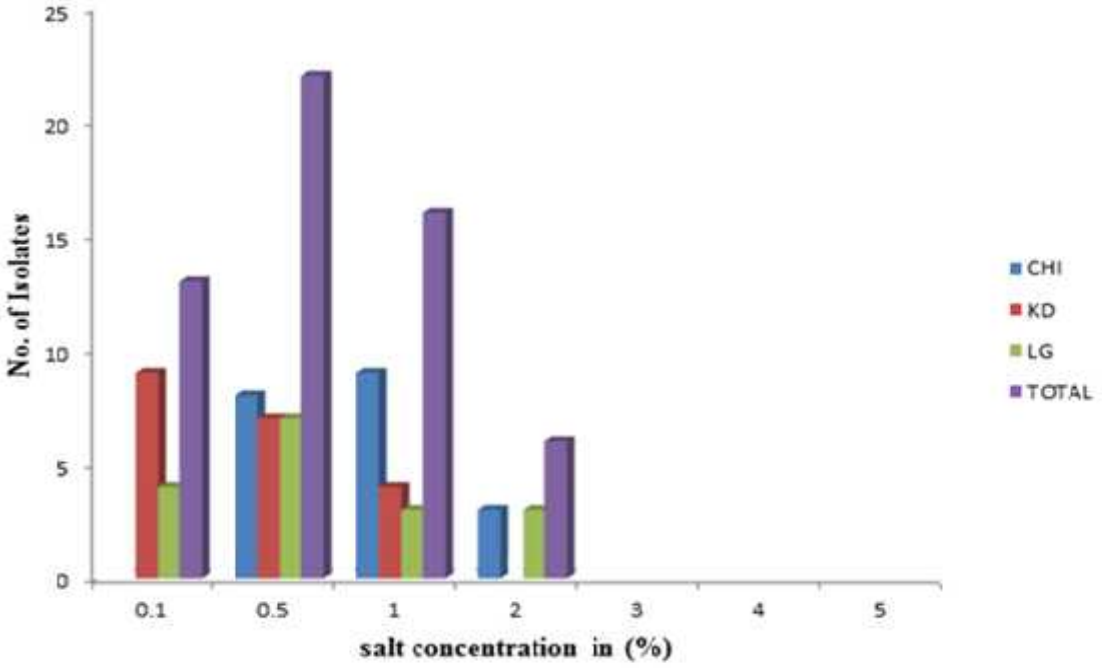


Figure 5: Effect of salt at different concentration on the growth of *Rhizobium* isolates.

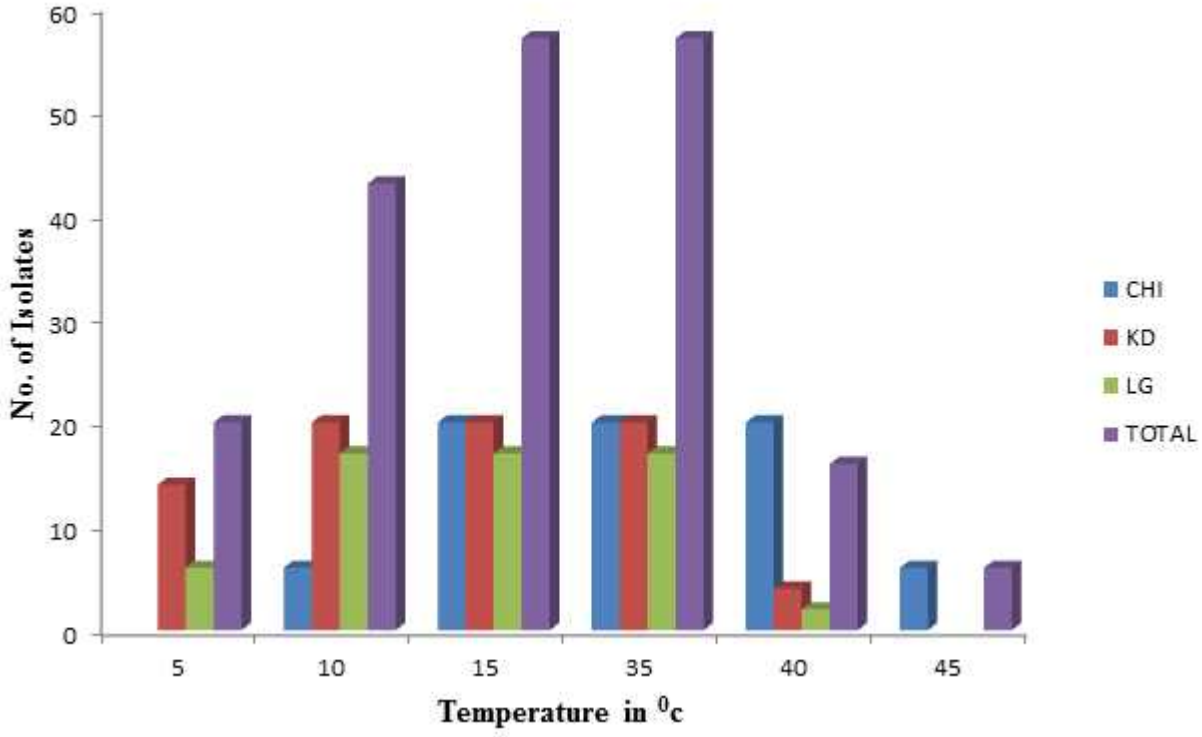


Figure 6: Effect of temperature on the growth of *Rhizobium* isolates.

5.4.5. Intrinsic antibiotic resistance

In the present investigation it was found that, all isolatesexhibited variations in their intrinsic antibiotic resistance to different concentrations and types of antibiotics (Table 3). Hundred percent of the tested isolates were resistant to Erythromycin and Ampicillin at 100µg and 125µg of concentrations, but at 150µg concentration of Erythromycin and Ampicillin antibiotics resistant of isolates were 67 % and 77%, respectively (Table 3). On the other hand, 100% of isolates were sensitive to Erythromycin and Ampicillin at 200µg concentration (Appendix A: Table 7). Whereas, resistant of isolates to Chloramphenicol antibiotics at 5µg and 10µg were 100%; at 15µg concentration 56% and at 25µg isolates were unable to grow (Table 3). Resistance of isolates to tetracycline at concentration of 5µg was 100%, at 10µg was 63% and at 15 µg and 25µg concentration of tetracycline was unable to grow.

Table 3: Tolerance of *Rhizobium* isolatesat different antibiotics with different concentrations.

Antibiotics	**Concentration (µg)**	**% of resistant isolates**
Erythromycin	100	100
	125	100
	150	67
Chloramphenicol	5	100
	10	100
	15	56
Tetracycline	5	100
	10	63
Ampicillin	100	100
	125	100
	150	77

5.5. Authentication or Symbiotic effectiveness of the isolates

5.5.1. Analysis of variance

Forty isolates obtained from presumptive test of faba bean nodules were assessed for their infectiveness and effectiveness of nitrogen fixation on Adet faba bean on sterile and acid treated sand in a pot experiment under greenhouse (Fig. 7). Out of these, only fifteen (37.5%) isolates formed nodules on the test faba bean root authenticating as *Rhizobium* (Table 4).

The results of analyses of variance showed that *Rhizobium* inoculation significantly ($P<0.05$) increased at all investigated parameters such as, number of nodules per plant, nodule fresh weight, nodule dry mass, shoot dry weight, shoot length, root biomass, plant total nitrogen and symbiotic effectiveness as compared to the standard and control treatments (Table 4).). Nodule number was recorded between 61.33 p^{-1} for isolate (KD-13) and 103 p^{-1} for isolate (LG-1) p^{-1} and the mean nodule numbers of isolates were found to be 71.4 per plant. The nodule number of the plant inoculated with standard *Rhizobium* was 81.33 p^{-1} (Table 4). On the other hand, nodule dry mass (NDM) was between 83.33mg $p^{-1}$for isolate (CHI-8) and 117.33 mg $p^{-1}$for isolate (KD-1) and the mean NDM recorded in this study was 86.8mg $p^{-1}$with Adet faba bean variety. The nodule dry mass of the plant inoculated with standard *Rhizobium* was 90.67mg p^{-1}(Table 4). Whereas, nodule fresh weight was recorded 116.0mg $p^{-1}$for isolate (KD-13) to 265.3 mg $p^{-1}$for isolate (KD-1) and the mean nodule fresh weight in this study was also 153.4mg $p^{-1}$with Adet faba bean variety.In this study, 200mg p^{-1}nodule fresh weight of the plant was recorded for standard *Rhizobium* from national soil laboratory, Addis Abeba (Table 4). Moreover, Shoot dry weight ranges from 2.257 g $p^{-1}$for isolate (CHI-2) and 6.970g $p^{-1}$for isolate (CHI-9) and the mean shoot dry weight recorded in this finding was 4.901g $p^{-1}$with Adet faba bean variety. The shoot dry weight of the plant in this study was 6.290g p^{-1} for standard *Rhizobium* from national soil laboratory, Addis Abeba (Table 4).

From this study it was shown that inoculation induces significant improvement in plant shoot length as compared to the control treatments. Plant shoot length exhibited by isolates KD-4 and LG-1 were 48.67cm and 48.67cm, respectively. The shoot length by standard treatment (isolate obtained from National Soil Laboratory, Addis Ababa) was 47.67 cm. There was one isolate (CHI-9) which showed similar shoot length as that of the standard (Table 4). The highest plant

shoot length 48.67cm was recorded for both isolate (KD-4) and (LG-1) inoculated with Adet faba bean variety (Table 4).

Root biomass was recorded between 0.73 g p $^{-1}$ for isolate (CHI-2) to 3.400 g p $^{-1}$for isolate (CHI-3) and the mean root biomasses of isolates were found to be 1.621g per plant (Table 4). All *Rhizobium* isolate inoculation resulted in a significant different at ($p<0.05$) in root biomass as compared to negative control. Approximately, 27% of isolates were display high root biomass as compared to standard *Rhizobium* from national soil laboratory, Addis Abeba (Table 4).

Plant total nitrogen ranging from 2.760 % for isolate (LG-1) to 4.292 % for isolate (KD-4) and the mean plant total nitrogen of isolates were 3.2241% (Table 4). In general, inoculation of *Rhizobium* isolates resulted in a significantly different at ($p<0.05$) in plant total nitrogen over negative treatments. In addition to the above data, there was also inoculation of *Rhizobium* isolates resulted in a significantly different at ($p<0.05$) in plant total nitrogen over positive and standard *Rhizobium* treatments except CHI-2 and standard, and LG-9 and nitrogen treated plant were not significant (Table 4).

Analysis of variance showed that inoculation was increased the plant total nitrogen as 31.3%, 29.4% and 85.7% over nitrogen treated plants, standard *Rhizobium* and negative treatment (plants without nitrogen sources and inoculation), respectively. The best nine isolates CHI-3, CHI-7, CHI-9, KD-1, KD-4, LG-1, LG-16, LG-17 and LG-9 with Adet faba bean variety, showed effectiveness ranging from 102.67% to 138.33% as compared to the nitrogen treated plants (Table 4). The best four isolates also CHI-3, CHI-9, KD-4, and LG-16 with Adet variety showed effectiveness of 127%, 138.33%, 135.67% and 129% respectively as compared to inoculants of standard *Rhizobium* which showed effectiveness of 124.67%. The standard *Rhizobium* (National Soil Laboratory, Addis Abeba) inoculated plant also showed effectiveness over nitrogen treated plants (Table 4).

Table 4: Nodulation and relative effectiveness of nitrogen fixation of R. leguminosarum bv. Viciae isolates of North Gondar (Chilga) and South Gondar (Farta and Lay Gayint) tested on Adet faba bean variety on sand using pot experimentunder greenhouse condition.

Isolates	NC	NN p^{-1}	NDM (mg)p^{-1}	NFW (mg) p^{-1}	SDW (g) p^{-1}	SL (cm)p^{-1}	RB (g)p^{-1}	PTN (%)	SE (%)
CHI-10	P	82.00cdefg	100.00bcdef	173.7bcde	4.693c	43.67bc	1.695cdefgh	3.639j	93.33c
CHI-2	P	70.33bcde	91.67bcde	149.0bcd	2.257ab	46.67cde	0.73 ab	3.051f	45.00ab
CHI-3	P	89.67efgh	94.00bcde	150.0bcd	6.389efg	46.00cde	3.400i	3.364h	127.00def
CHI-7	P	94.00fgh	110.67ef	231.0ef	5.158cde	43.67bc	1.652bcdefg	3.671k	102.67cd
CHI-8	P	69.00bcd	83.33b	162.0bcd	4.453c	43.67bc	1.197bcdef	2.969e	87.67c
CHI-9	P	95.67gh	105.67def	141.0bc	6.970g	47.67de	2.190gh	4.160m	138.33f
KD-1	P	90.00fgh	117.33f	265.3f	5.427cde	42.00b	1.853efgh	3.419i	110.33cdef
KD-13	P	61.33b	83.67b	116.0b	4.644c	43.33bc	1.902efgh	2.848c	92.33c
KD-19	P	63.67bc	88.33bcd	140.3bc	2.735 b	46.00cde	0.817abcd	4.111 l	54.33b
KD-4	P	88.00defgh	102.00bcdef	149.0bcd	6.808fg	48.67e	1.753defgh	4.292n	135.67ef
KD-9	P	64.67bc	85.67bc	149.0bcd	2.871b	43.67bc	0.983abcde	3.359h	56.67b
LG-1	P	103.00h	104.33cdef	153.0bcd	5.747cdefg	48.67e	2.437gh	2.760b	114.33cdef
LG-16	P	86.33defgh	101.00bcdef	186.0cde	6.481efg	43.00bc	2.647hi	3.200g	129.00def
LG-17	P	69.33bcd	99.00bcdef	186.7cde	5.496cdef	44.33bcd	0.758abc	3.677k	109.00cde
LG-9	P	76.00bcdef	105.67def	208.3def	5.535cdef	46.00cde	1.983fgh	2.925d	110.33cdef
Standard	P	81.33cdefg	90.67bcd	200.0cdef	6.290defg	47.67de	2.020fgh	3.029f	124.67def
N+		0.00a	0.00a	0.00a	5.040cd	43.33bc	1.070bcdef	2.947de	100.00cd
N-		0.00a	0.00a	0.00a	1.230a	22.00a	0.080a	0.612a	24.33a
Grand Mean		71.4	86.8	153.4	4.901	43.89	1.621	3.2241	97.5
CV (%)		16.4	13.7	26.1	14.4	4.6	30.7	0.5	15.5
LSD (0.05)		19.37	19.70	66.28	1.1693	3.312	0.8238	0.0277	25.05

P= pink, NC= nodule color, NN= nodule number, NDW= nodule dry weight, NFW= nodule fresh weight, SDW= shoot dry weight, SL=shoot length, RB= root biomass, PTN=plant total nitrogen, SE=symbiotic effectiveness, p^{-1}=per plant. N- = without chemical and inoculation, N+ =with optimum amount of N fertilizer, CV= Coefficient of variation, LSD= least significant difference. 0 = not found. Means within a column followed by the same letters are not significant at $p< 0.05$.

With Nitrogen plantRhizobium inoculated plant

Standatrd*Rhizobium* (green pots) &Rhizobium inoculant (red pot)

Our *Rhizobium* (red pots)

Comparasion b/n Standatrd*Rhizobium* With out nitrogen and inoculation plant

(Green pots)&Our *Rhizobium* (Red pots)

Figure 7: Some representative authentication tests on sand culture using pot experiment.

5.5.2. Correlation analysis

Nodule number was found to be positively correlated with nodule fresh weight (r= 0.469 P <0.01) and strongly positive correlated with nodule dry mass (r=0.677 P <0.01), shoot dry weight (r = 0.591 P <0.01), root biomass (r=0.561 P <0.01) and symbiotic effectiveness (r=0.586 P <0.01) (Table 5). Shoot dry weight was found to be positively correlated with Nodule dry mass (r=0.393 P <0.01), strongly positive correlated with root biomass(r=0.614 P <0.01) and symbiotic effectiveness(r= 0.994 P <0.01) (Table 5).

Shoot length was found to be positively correlated with nodule dry mass (r= 0.396 P<0.01) and with nodule fresh weight (r= 0.391P <0.01) (Table 5).Nodule fresh weight was negatively correlated with soil nitrogen (r=-0.318 P <0.05) and there was also strongly positive correlation among nodule fresh weight (r=0.775 P <0.01), root biomass (r=0.610 P <0.01) (Table 5).

Table 5: Correlation coefficients among investigated parameters in faba bean; nodule number, nodule dry mass, nodule fresh weight, shoot dry weight, shoot length, root biomass, plant total nitrogen, symbiotic effectiveness , soil PH, and soil Nitrogen.

	NN p^{-1}	NDM (mg) p^{-1}	NFW (mg) p^{-1}	SDW(g) p^{-1}	SL (g) p^{-1}	RB (g) p^{-1}	PTN (%)	SE (%)	Soil pH	Soil N (%)
NN p^{-1}	1	0.677**	0.469**	0.591**	0.136ns	0.561**	0.115ns	0.586**	0.106ns	-0.285ns
NDM (g)p^{-1}	0.677**	1	0.775**	0.393**	0.396^{**}	0.300*	0.158ns	0.416**	-0.055ns	-0.270ns
NFW (g) p^{-1}	0.469**	0.775**	1	0.217ns	0.391^{**}	0.099ns	0.018ns	0.239ns	-0.060ns	-0.318*
SDW (g) p^{-1}	0.591**	0.393**	0.217ns	1	0.098ns	0.614**	0.181ns	0.994**	-0.088ns	-0.205ns
SL (cm) p^{-1}	0.136ns	0.396^{**}	0.391^{**}	0.098ns	1	0.124ns	0.190ns	0.082ns	0.070ns	-0.081ns
RB (g) p^{-1}	0.561**	0.300*	0.099ns	0.614**	0.124ns	1	0.144ns	0.610**	-0.053ns	-0.236ns
PTN (%)	0.115ns	0.158ns	0.018ns	0.181ns	0.190ns	0.144ns	1	0.178ns	0.284ns	0.202ns
SE (%)	0.586**	0.416**	0.239ns	0.994**	0.082ns	0.610**	0.178ns	1	-0.095ns	-0.202n
Soil pH	0.106ns	-0.055ns	-0.060ns	-0.088ns	0.070ns	-0.053ns	0.284ns	-0.095ns	1	-0.410**
Soil N (%)	-0.285ns	-0.270ns	-0.318*	-0.205ns	-0.081ns	-0.236ns	0.202ns	-0.202ns	-0.410**	1

** = significant at P < 0.01, * = significant at P < 0.05 and ns = not significant at p <0.05

NN=nodule number, NDM (g)=nodule dry mass, NFW (g)=nodule fresh weight, SDW (g)=shoot dry weight, SL (cm)=shoot length, RB (g)=root biomass, PTN (ppm)= plant total nitrogen, SE (%)= symbiotic effectiveness, Soil N(ppm)= soil nitrogen andp^{-1}=per plant.

5.6. Hierarchal cluster analysis

Hierarchal cluster analysis for fifty seven isolates of *Rhizobium* based on their physiological characteristics including carbon source utilization, pH tolerance, salinity tolerance, temperature tolerance anddifferent antibiotic tolerance at different concentrations (Appendix A Table 3, 4, 5, 6 and 7) were used to construct a dendrogram using average linkage clustering method between groups. Based on the above parameters, *Rhizobium* isolates from different North and South Gondar locations essentially grouped in to six main clusters and the individual main cluster contains sub clusters to analyze their similarity using Pearson's coefficient on the dendrogram (Fig. 8).

Cluster 1 contained 7 isolates out of 57 isolates of *Rhizobium* in this study. This cluster is further subdivided into two sub clusters (C-1A and C-1B). Sub cluster C-1A includes CHI-1, CHI-14, CHI-16 and CHI-20 isolates from Chilga and sub cluster C-1B includes CHI-8 and CHI-15 isolates from Chilga and the only isolate from Kimir Dingay in this sub cluster was KD-3 (Fig. 8). Cluster 2 comprised 12 isolates from 57 isolates of *Rhizobium*, which further grouped in to two sub clusters (C-2A and C-2B). Sub cluster C-2A again sub divided into two sub clusters (C-2A1 and C-2A2) which includes 4 isolates from 12 and sub cluster C-2B grouped in to two sub clusters (C-2B1 and C-2B2) which includes 8 isolates out of 12. Sub cluster C-2A1 includes CHI-13 and CHI-17 isolates, C-2A2 also includes CHI-3 and CHI-9 isolates from Chilga sites. However, sub cluster C-2B1 includes CHI-2, CHI-10 and CHI-11 isolates, C-2B2 in this sub cluster also includes CHI-4, CHI-5, CHI-6, CHI-7 and CHI-19 isolates from Chilga site (Fig. 8).

Cluster 3 consisted 5 isolates, which further sub divided into two sub clusters (C-3A and C-3B). Sub cluster C-3A includes LG-7, LG-10, LG-14 isolates from Lay Gayint and CHI-12 isolate from Chilga and sub cluster C-3B contains only isolate CHI-18 from Chiga (Fig. 8) Cluster 4 contained 14 isolates, which further sub divided into two sub clusters (C-4A and C-4B). Sub cluster C-4A includes KD-7, KD-14 and KD-18 isolates from Kimir Dingay. On the other hand, sub cluster C-4B again sub divided into two sub clusters (C-4B1 and C-4B2) and sub cluster C-4B1 includes isolate KD-15 from Kimir Dingay, LG-12, LG-6 and LG-8 isolates from Lay Gayint sites and sub cluster C-4B2 includes KD-1, KD-13, KD-9, KD-4, KD-3, KD-8 and KD-20 isolates from Kimir Dingay sites(Fig. 8).

Cluster 5 consisted 12 isolates, which further sub divided into two sub clusters (C-5A and C-5B). Sub cluster C-5A again sub divided into two sub clusters (C-5A1 and C-5A2) and sub cluster C-5A1 includes KD-12,KD-16,KD-11 isolates from Kimir Dingay sites, LG-1, LG-9 and LG-17 isolates from Lay Gayint sites. On the other hand, sub cluster C-5A2 includes LG-5 and LG-13 isolates from Lay Gayint and isolate KD-2 from Kimir Dingay sites. Whereas, Sub cluster C-5B includes isolate KD-19 from Kimir Dingay site and LG-2 and LG-15 isolates from Lay Gayint sites (Fig.8). Cluster 6 comprised 7 isolates out of 57, which further sub divided into two sub clusters (C-6A and C-6B). Sub cluster C-6A includes isolate LG-11 from Lay Gayint, KD-5, KD-6 and KD-10 isolates from Kimir Dingay. Whereas, sub cluster C-6B includes isolate KD-17 from Kimir Dingay, LG-4 and LG-16 isolates from Lay Gayint sites (Fig. 8).

The highest similarity were computed between isolate (KD-4)from Kimir Dingay and (LG-3) from Lay Gayint in cluster 4 and isolate (KD-12) from Kimir Dingay and (LG-9) from Lay Gayint in cluster 5 which were nearly100% similar(Fig. 8). This 100% similarity indicates, the isolates were isolated from the same soil pH, temperature, salinity, resistance of different antibiotics at different concentrations and carbon source utilization (Appendix A Table 3, 4, 5, 6 and 7). On the other hand, CHI-6 and CHI-19 isolates from Chilga in cluster 2, CHI-14 and CHI-20 isolates from Chilga in cluster 1 and KD-6 and KD-10 isolates from Kimir Dingay in cluster 6 were revealed 88% similarity on the dendogram(Fig. 8). Whereas, the lowest similarity was computed between isolate CHI-15 from Chilga and isolate KD-6 from Kimir Dingay which presented in to two different clusters (i.e. CHI-15 in cluster 1 and KD-6 in cluster 6)(Fig. 8). This lowest similarity shows, the isolates were isolated from different soil pH (CHI-15 ranging 5-9 and KD-6 between 5.5-8), temperature (CHI-15 between 15-40^{0}c and KD-6 ranging from 5-35^{0}c), salt concentration (CHI-15 at 0.5 % w/v and KD-6 at 1% w/v), carbon utilization (CHI-15 uses 87% and KD-6 uses 100% of tested carbon sources) and different antibiotics at different concentrations (Appendix A Table 3, 4, 5, 6 and 7). Similarity increases as we read from right to left on the denogram, based on Pearson's correlation coefficient value (Fig. 8).

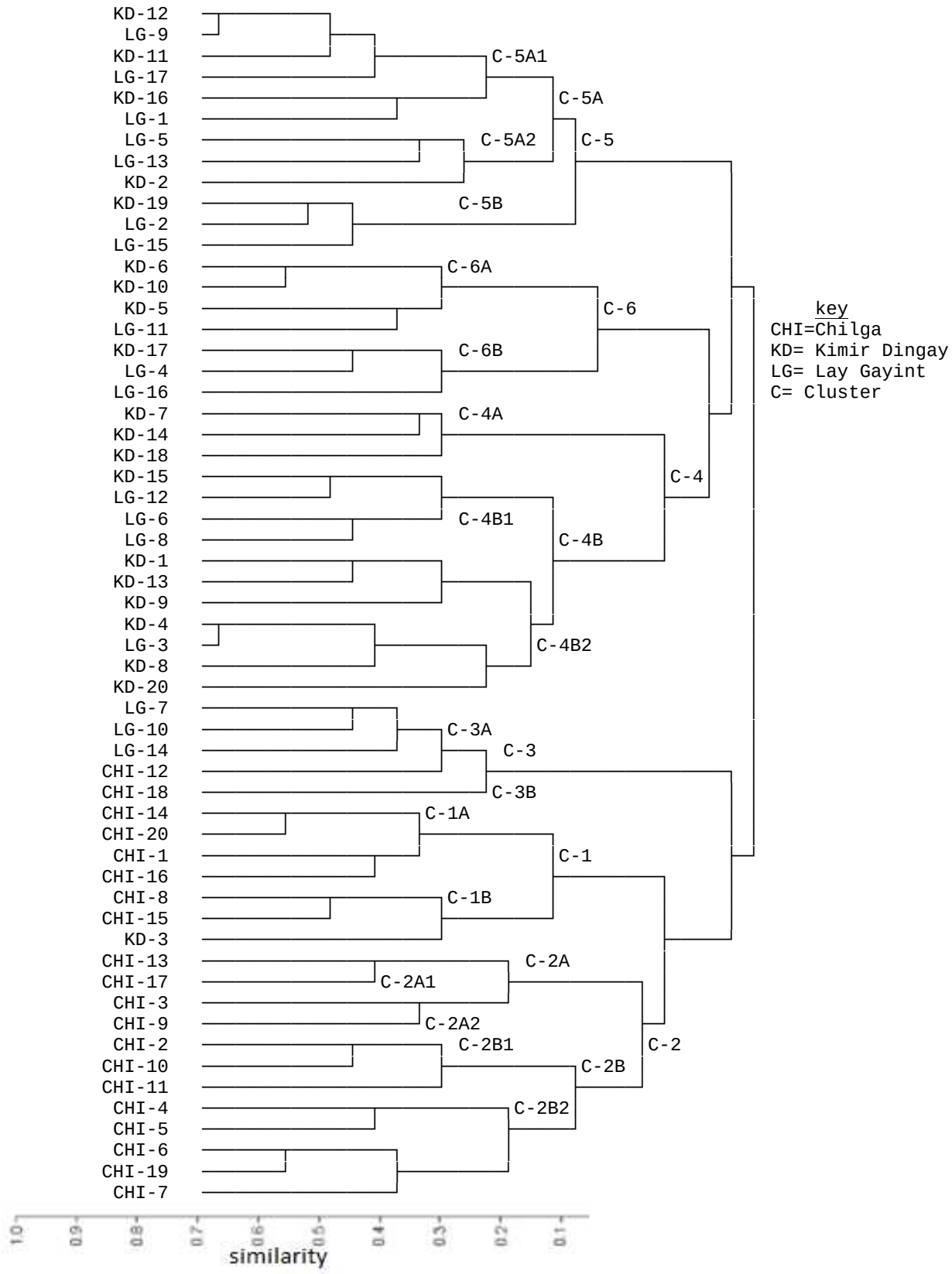


Figure 8: Dendrogram showing the similarity using Pearson's coefficient among the physiological characterization of fifty seven Rhizobium isolates grouped based on average linkage hierarchical cluster analysis between groups.

6. DISCUSSION

Polyphasiccharacterization tests were performed to compare the responses of fifty seven*Rhizobium leguminosarum*bv. *viciae*isolates.All isolates found gram-negative, rod-shaped, milky-white opaque color of colonies, circular shape, regular borders and raised, showing intermediate to high production of mucus after 3 to 5 day of growth on YEMA medium at 30^0C. These were the characteristics of Rhizobia (Lupwayi and Haque, 1994). Furthermore, the colony diameter of all isolates of *Rhizobium leguminosarumbvviciae* was within the range of 1.5 - 4.5mm after 3 to 5 days of incubation at 30 ^{o}C (Appendix A: Table 2). According to the previous report of Jordan (1984) *Rhizobiumleguminosarum* isolates usually showed colony size between 2-4 mm in diameter. In the present study 95 % of isolates fall to this group.

All the isolates changed the YEMA-BTB medium to yellow color and did not absorb Congo red on YEMA-CR media indicated that all the isolates are acid producer and fast growing rhizobia. Similar classification has been done by Jordan (1984). This color formation is due to the utilization of the sugar component of the medium for their growth (Tan and Broughton, 1981). This finding was similar with the previous work of Aynabeba *et al.* (2001) indicating that many *Rhizobium* strains isolated from each sampling field in Semen Shewa were fast growing and acid producing. All the strains were gram negative and rod shaped as revealed by Gram's staining technique. The results of the present study was similar reports obtained by Singh *et al.*(2008); Tagelsir and Mohamed (2015).

The isolates were found to utilize 86 to 100% of the tested carbohydrates which were served as good sources for the growth of rhizobial (Table 2). According to Somasegaran and Hoben, (1994) carbohydrate utilization properties have taxonomic significance for rhizobial strains. *Rhizobium spp*. utilizes a wide range of carbohydrates as reported by many researchers (Hafeez *et al*, 1995; Maatallah *et al.*, 2002). The ability to utilize a wide range of carbon sources have an ecological advantage in colonizing the soil or rhizosphere as compared to strains having a degree of specificity in their requirements (Sukrita and Chakrabarti, 1981). Similar findings have been reported on carbohydrate metabolism of *R. leguminosarum bv. viciae* (Hafeez *et al.*, 1995; Zerihun and Fassil, 2011).

Most isolates showed growth on a wide range of moderate acidity and alkalinity (5 to 9 pH) and did not grow at the tested pH value of 4 and 4.5 (Fig. 4).This result is in agreement with the previous finding of pH tolerance that *Rhizobium leguminosarum bv. viciae* strains showed sensitivity to low pH and grow well on near neutral and basic pH values (Zerihun and Fassil, 2011). Similar finding was reported by Keneni *et al*. (2010) that, no *Rhizobium* isolates were able to grow at pH of the medium adjusted to 4 and 4.5 including the exotic strains. Strains of rhizobia differ markedly in their tolerance to acidic pH (Ballen *et al.*, 1998; Zahran, 1999). Even within the same species, strains differ considerably in their tolerance to acidity in culture media (Glenn and Dilworth, 1994).

None of the isolates from Chilga, 9 isolates (KD-1, KD-4, KD-7, KD-8, KD-9, KD-13, KD-14, KD-15 and KD-20) from Kimir Dingay and 4 isolates (LG-3, LG-6, LG-8 and LG-12) from Lay Gayint soil sites were grown on the medium containing 0.1% of NaCl (Fig. 5). With regard to all isolates (i.e. 20) from Chilga did not show growth on the medium containing 0.1% of NaCl. On the other hand, in the report of Zerihun and Fassil, (2011) all the isolates (i.e. 17) collected from Northern Gondar, Ethiopia, grew on the medium containing 0.1% NaCl. Whereas, 6 (10.5%) of the isolates grew on the medium containing 2% NaCl including, 3 isolates (CHI-4, CHI-12 and CHI-18) from Chilga and 3 of them (LG-7, LG-10 and LG-14) from Lay Gayint soil sites (Fig. 5). On the other hand, all *Rhizobium leguminosarum* collections were inhibited at 2% NaCl concentration on the report of Jordan, (1984). This finding is more or less similar with isolate AURF128 which found to be tolerant to NaCl concentrations of 2 % out of 17 isolates isolated from Northern Gondar, Ethiopia by (Zerihun and Fassil, 2011). All isolates from all study areas did not tolerate the medium containing 3-5 % NaCl concentrations (Fig. 5). This finding is contrary to previous reports that, in general, fast growing *Rhizobium* grew well at NaCl concentration between 3 and 5% (Abdul-wahab and Zahran, 1979; Zerhari *et al.*, 2000).

All of rhizobial isolates showed growthat 15 and 35°C.According to Lindstrom and Lehtomaki(1988) report, the temperature tolerance is within the range of 32.5 to 34.5°Cfor *Rhizobium leguminosarum bv. viciae* HAMBI499, HAMBI 1125 and MPI 6001isolates isolated from faba bean and field pea from USA, UK and the Netherlands. There was 6 (10.5%) of the isolates showed growth at temperature of 45^0C which were collected from Chilga only (CHI-3, CHI-9, CHI-11, CHI-13, CHI-17 and CHI-118) (fig. 6).This finding is nearly similar with

survival of 10 strains out of 23 strains at a temperature of 45 ^{o}C and 7% of both fast growing and slow growing rhizobia nodulating chickpea (*Cicer arietinum*) showed survival at 45^{o}C(Maatallah *et al.*, 2002) and contrast to the previous report by Jordan, (1984), revealed that the maximum growth temperature for *Rhizobium leguminosarum* was 38^{0}C. Somasegaran and Hoben (1994) reported that the optimal growth for most strains of Rhizobia occurs at a temperature range of 25-30^{o}C. Four strains nodulating chickpea remain unaffected in their mean doubling time at a temperature of 40^{0}C and 7 strains showed 3-5fold increase (Khokhar *et al.*, 2001). The upper limit for rhizobial growth ranges between 32 and 47^{0}C though tolerance varies among species and strains (La Favre and Eagleshman, 1986). Physiological and genetic modifications in bacteria such as plasmid deletion and genomic rearrangements may occur in soils subject to high temperature tolerance (Hungria and Vargas, 2000).

According to Purcino *et al*.(2000) the survival, persistence and competitiveness of the rhizobial strains are the major determinant factors for successful use of the rhizobia as inoculant. To find out these features, the inoculated strains must be distinguished from the other Rhizobia present in the soil. A large method of identification of the inoculants was performed, but use of intrinsic antibiotics resistance is the simplest and most commonly used method for strain identification (Kucuk *et al*., 2006).

In the present investigation it was found that, all isolates exhibited variations in their intrinsic antibiotic resistance to different concentrations and types of antibiotics (Table 3). Hundred percent of the tested isolates were resistant to Erythromycin and Ampicillin at 100µg and 125µg of concentrations, but at 150µg concentration of Erythromycin and Ampicillin antibiotics resistant isolates were 67 % and 77%, respectively (Table 3). On the other hand, 100% of isolates were sensitive to Erythromycin and Ampicillin at 200µg concentration (Appendix A Table 7). This finding was similar to the antibiotic resistance of the isolates of *Rhizobium leguminosarum bv. viciae* reported by Zerihun and Fassil (2011). In the present study the resistannce capacity of isolates to Chloramphenicol antibiotics at 5µg and 10 µg were 100%. But their resistance at 15µg concentration was 56% whereas the isolates were unable to grow at 25µg (Table 3). Resistances of isolates to Tetracycline at concentration of 5µg were 100%. But their resistances at 10µg were 63% and at 15 µg and at 25µg concentration of tetracycline all were sensitive. This result showed that as concentrations of antibiotics increase, resistance of isolates

decreased and the finding was in agreement with previous report by Zerihun and Fassil, (2011). Abdel -Wahab *et al.*(1976) reported that several *Rhizobium trifolii* strains were resistant to Penicillin and sensitive to Tetracycline and Chloramphenicol and similar observations were made by the work of Hagedorn (1979) on *R.trifolii*. Kucuk and Kivanc (2008) reported that broad ranges of intrinsic antibiotic resistance pattern were found in strains isolated from chickpea nodules in Eskisehir areas of Turkey with a high level of resistance shown against Streptomycin, Erythromycin, Kanamycin, Penicillin and Chloramphenicol.

The ability to form nodules along with the subsequent capacity of fixing nitrogen is widely used as means of assessing the association between rhizobia and respective hosts (Brockwell *et al*., 1995). Some findings suggests that those isolates that failed to nodulate their parent host are either unwanted soil bacteria that penetrated the nodules of the host (Johnston and Beringer, 1976) or *Rhizobium* may lost their nodulation capacity due to a loss of plasmids (Segovia *et al*., 1991; Zhang *et al*., 2001). Van Berkum *et al*., (1995) indicated that, on the basis of host plant specificity for nodulation and infection of the symbiotic associations of species were generally assumed to be *Rhizobium leguminasorium bv viciae.*

The results of analyses of variance showed that *Rhizobium* inoculation significantly ($P<0.05$) increased at all investigated parameters such as, number of nodules per plant, nodule fresh weight, nodule dry mass, shoot dry weight, shoot length, root biomass, plant total nitrogen and symbiotic effectiveness as compared to the standard and control treatments (Table 4). This finding is similar with Dereje *et al*. (2015) symbiotic effectiveness of faba bean nodulating on sand culture and Anteneh (2012) symbiotic effectiveness of *Rhizobium leguminosarum var. Viciea* nodulating lentil in Ethiopian. Somasgaran and Hoben (1994) reported that, bacterial inoculation of plants formed red and pink nodules, which indicated for the formation of effective nodules with effective nitrogen fixation and for the presence of leghemoglobin (Amare*et al*., 1995).

In the present study the nodulating isolates showed difference with nodule number. The variation of nodulation is the result of low rhizobial density, inconsistence of the *Rhizobium* and edaphic factors that delay the effectiveness of the rhizobial isolates (Zahran, 1999; Slattery and Pearce, 2002; Kiros and Singh, 2006).

The isolates also showed difference in nodule dry mass with Adet faba bean (Table 4), which were higher than isolate difference between 37 to 111 mg p $^{-1}$on sand culture reported by Zerihun and Fassil (2011). Similarly, we observed relatively higher nodule dry weight value than the 79mg p $^{-1}$and 78mg p $^{-1}$which were reported by Dereje *et al*. (2015) and Girmaye *et al*. (2014) respectively, but lower than145mg p $^{-1}$which was reported by Anteneh (2012).

In the present finding, the result showed that there is a 3 fold difference between the isolates accumulating the lowest and highest shoots dry weight in the host which is a very good indicator of effectiveness in nitrogen fixation of legumes (Sorwill and Myaton, 1986; Somasegaran and Hoben 1994; Date, 1993 in Purchino, 2000; Peoples *et al.*, 2002). Therefore, we perceive higher shoot dry weight value than shoot dry weight difference ranging from 0.4 g p $^{-1}$from (AUFR127) to 2.3 g per plant from (AUFR 124) on sand culture reported by Zerihun and Fassil (2011) from North Gondar, 0.54 g to 1.95 g p $^{-1}$by Girmaye (2009) isolates from acidic soils of Wollega and 0.38 g to 1.95 g p $^{-1}$by Getaneh (2008) isolates from western Shewa and Hararghe.

In the present study, shoot length improvement was the best as compared with the results of Dereje *et al*. (2015) study on faba bean inoculation with Degaga variety with 43.3 cm for isolate HUGAVf1 collected from acidic soils of Ethiopia, which was 32.94% over the negative control and 16.9% over the positive controls. The result was also better than with the result of Anteneh (2012) study on faba bean inoculation with Degaga variety with 49.7 cm for isolate NSFBR-48 collected from Central Ethiopia, which was shown noticeable improvement in shoot height 51% over negative control and 14% nitrogen treated plants. This improvement of shoot length could be regard as; the rhizobia may increase plant growth by providing products through nitrogen fixation (Kumar *et al*., 2014).

In this pot experiment on sand culture during authentication, application of mineral nitrogen fertilizer did not improve growth and development of plants; instead it delayed and inhibited nodulation and effectiveness of nitrogen fixation potential of *Rhizobium* isolates. The result was in agreement with the addition of nitrogen fertilizer has been a negative effect on the nodulation and nitrogen fixation *Rhizobium* isolates (Chemining'wa *et al*., 2004; Crews *et al*., 2004).In Ethiopia, Similar application of inorganic nitrogen fertilizer has not shown any significant yield difference on lentil (Angaw and Asnakew, 1994).

According to the percentage differences of shoot dry weight of inoculated and nitrogen-fertilized plantsa measure of effectiveness (Date, 1993 in Purcinho, 2002), 80% of the isolates were found to be highly effective, 13 % were effective and 7 % was lowly effective nitrogen fixers (Table 4). The data show that, more than 93% of the sampling sites in North and South Gondar were highly effective and effective *Rhizobium* on the sand culture. This result reveals, the effectiveness of isolates were relatively high with the finding of Zerihun and Fassil (2011) where 80% of isolates from Northern Gondar were effective, but contrary to the previous finding of Desta and Angaw (1987) were only 11% of the isolates from Central Shewa were effective. According to Van Berkum *et al*. (1995) and Brockman and Bezdicek (1989) report, variation in effectiveness of isolate was also found to be widespread in Ethiopia and USA respectively.

According to Graham O'Hara *et al*.(2002) report, the first parameter for a *Rhizobium* used as inoculant or biofertilizer is, it must be superior and highly effective in nitrogen fixing ability forming symbiotic association with the host legume. The best nine isolates CHI-3, CHI-7, CHI-9, KD-1, KD-4, LG-1, LG-16, LG-17 and LG-9 with Adet faba bean variety, showed effectiveness rangingfrom 102.67% to 138.33% as compared to the nitrogen treated plants (Table 4). More highly effectiveness of isolates indicates the fact that the production of plant growth promoting hormone (Erum and Bano, 2008). The best four isolates also CHI-3, CHI-9, KD-4, and LG-16 with Adet variety showed effectiveness of 127%, 138.33%, 135.67% and 129% respectively s compared to inoculants of standard *Rhizobium* which was shown effectiveness of 124.67% (Table 4).

In this study, more highly effective isolates were obtained compared to other investigator reports. According to Dereje *et al*. (2015) finding 56% the isolates were highly effective in both Degaga and Dosha varieties collected from acidic soils of Ethiopia, Girmaye *et al*. (2014) revealed that 16% of the isolates of faba bean were highly effective collected from acidic soils of Wollega, Western Ethiopia and Anteneh (2012) result shows 20.9% of isolates were very effective collected from major lentil growing areas of Ethiopia. Generally, the results of this study indicates that, screening of local *Rhizobium* isolates gives paramount importance for enhancement of dinitrogen fixation takes place in faba bean.

Shoot dry weight was found to be positively correlated with Nodule dry mass ($r=0.393$ $P<0.01$), strongly positive correlated with root biomass($r=0.614$ $P<0.01$) and symbiotic effectiveness(r=

0.994 P <0.01) (Table 5).Shoot dry weight and nodule dry weight are usually positively correlated and Somasegaran and Hoben(1994) finding reveals that, shoot dry weight was used regularly as an indicator of relative symbiotic effectiveness.Shoot length was found to be positively correlated with nodule dry mass (r= 0.396 P<0.01) and with nodule fresh weight (r= 0.391P <0.01) (Table 5).Nodule fresh weight was negatively correlated with soil nitrogen (r=-0.318 P <0.05) and there was also strongly positive correlation among nodule fresh weight (r=0.775 P <0.01), root biomass (r=0.610 P <0.01) (Table 5).A similar result was reported on lentil, using growth pot experiment by Anteneh (2012) and Zafar-ul-Hye *et al*. (2007) and groundnut (Nguyen *et al*., 2002). Many research findingreveals that nodulation status positively correlated with plant tissue nitrogen, symbiotic effectiveness and shoot biomass or dry weight (Mnalku *et al*., 2009; Atici *et al*., 2005).

7. CONCLUSION AND RECOMMENDATIONS

7.1. CONCLUSION

In the present study, most of our isolatesdisplayed abundant diversity in their response to morphological and physiological characteristics. The growth of isolatesat stressed laboratory conditions (at pH 5 and 8; 2 % NaCl; 5 and 45^{o}C) indicated their significance in contributing biologically fixed nitrogen to stressful ecosystems. Isolates that show tolerance to both salinity and pH extremes may be potential candidates for inoculum production,this in turn effective for nitrogen fixation in combination with the available cultivars. Several carbon sources have been utilized by isolates obtained in this study. This ability has an ecological advantage in colonizing the soil or rhizosphere. Hence, isolation and polyphasic characterization studies were essential for the selection of strains adapted to marginal edaphic- climatic conditions that can perform better for nodulation of plant growth and provides information about their genetic diversity.

Inoculation of isolates significantly increased at all investigated parameters such as number of nodules per plant, nodule fresh weight, nodule dry mass, shoot dry weight, shoot length, root biomass, plant total nitrogen and symbiotic effectiveness as compared to the standard and control treatments.About 80% of the isolates collected from major faba bean growing areas of North and South Gondar, Ethiopia were found to be highly effective. About 13 % were effective, only one isolate (7 %) from Chilga area was categorized as less effective and none of isolates grouped as ineffective.

The best nine effective isolates were selected over nitrogen treated plants with Adet faba bean variety from Chilga (3), Kimir Dingay (2) and Lay Gayint (4) study areas.The best four effective isolates, 2 of them from Chilga, 1 from Kimir Dingay and 1 from Lay Gayint were also selected as compared to the standard *Rhizobium* isolate from National Soil Laboratory, Addis Abeba.In general,inoculation of selected *Rhizobium*isolatesshowed better shoot dry weight enhancement over nitrogen treated plants of Adet faba bean on sand culture using pot experiment under controlled greenhouse condition.

7.2. RECOMMENDATIONS

Based on the finding of this study, the following recommendations were suggested:

- There is a need for detailed further study on biological nitrogen fixation practices to ensure that valuable knowledge for farmers on some nitrogen fixer bacteria and thereby to utilize such environmental friendly fertilizer sources.
- Research should be undertaken on the symbiotic effectiveness of this*Rhizobium* on other crops such as cereals in different parts of Ethiopia.
- Further investigations are required for effective isolatesto be tested under greenhouse and field condition on soil culture to assess their competitiveness ability, adaptability to the wide edaphic condition and survival and colonization within soil.
- Further research using molecular approaches (protein and DNA analysis) is required to characterize the *Rhizobium*isolates.

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9. APPENDIX

Appendix A: table 1. Induction of nodulation (Presumptive test) of isolates from Chilga, Farta (kimir dingay) and Lay Gayint woredas soil sites.

Isolates	Nodule number	Nodule fresh Weight (g)	Nodule color	Shoot height (cm)	Leaf color
CHI-1	89	0.28	W	29	G
CHI-2	72	0.18	P	29	G
CHI-3	67	0.21	P	25	G
CHI-4	142	0.95	W	25	G
CHI-5	129	0.87	P	25.6	G
CHI-6	120	0.82	P	25	G
CHI-7	142	0.92	P	28	G
CHI-8	39	0.21	P	31	G
CHI-9	112	0.85	P	30	G

CHI-10	108	0.81	P	27	G
CHI-11	172	1.05	P	32.5	G
CHI-12	135	0.89	P	23	G
CHI-13	95	0.41	P	26	G
CHI-14	105	0.92	W	29	G
CHI-15	108	1.01	P	28.7	G
CHI-16	156	0.96	P	27	G
CHI-17	142	0.92	W	24.5	G
CHI-18	82	0.51	P	25.5	G
CHI-19	108	0.99	P	27	G
CHI-20	97	0.86	W	25.5	G
KD-1	92	0.62	P	23.7	G
KD-2	38	0.2	P	23.5	G
KD-3	7	0.08	P	21.5	G
KD-4	158	1.08	P	31	G
KD-5	49	0.35	P	26	G
KD-6	82	0.45	P	27	G
KD-7	118	1.04	P	31	G
KD-8	92	0.71	W	26	G
KD-9	94	0.56	P	32	G
KD-10	56	0.42	W	27.5	G
KD-11	87	0.56	P	24.7	G
KD-12	68	0.26	P	27.5	G
KD-13	85	0.51	P	27	G
KD-14	89	0.53	P	25.7	G
KD-15	96	0.71	P	27.5	G
KD-16	95	0.34	P	28	G

Appendix A: table 1: continue...

KD-17	59	0.38	P	21.7	G
KD-18	78	0.43	P	31.6	G
KD-19	105	0.82	P	24	G
KD-20	107	1.02	P	25	G
LG-1	122	0.73	P	26	G
LG-2	147	0.84	P	29	G
LG-3	88	0.62	W	26.5	G
LG-4	8	0.09	P	13.5	G
LG-5	32	0.27	P	18	G
LG-6	127	0.72	P	20.3	G
LG-7	42	0.27	P	22.5	G
LG-8	89	0.67	P	26	G

LG-9	18	0.41	P	17	G
LG-10	102	0.91	P	22	G
LG-11	51	0.35	P	22.5	G
LG-12	49	0.29	W	22	G
LG-13	65	0.43	P	28	G
LG-14	73	0.51	W	27	G
LG-15	178	1.09	P	28	G
LG-16	167	1.05	P	25.3	G
LG-17	152	1.08	P	24.3	G

CHI= Chilga, KD= Kimir Dingay, LG=Lay Gayint, P= Pink, W= White and G= Green

Appendix A: table 2. Morphological and Biochemical Characteristics of Rhizobia isolated from faba bean grown on YEMA at 30ºC.

Isolates	Color in Congo red	Color in BTB medium	Colony size (mm)	Colony shape	Colony Color	Gram reaction
CHI-1	White	Yellow	2.5	Circular	White	-ve, rod
CHI-2	White	Yellow	2.0	Circular	White	-ve, rod
CHI-3	White	Yellow	4.0	Circular	White	-ve, rod
CHI-4	White	Yellow	3.0	Circular	White	-ve, rod
CHI-5	White	Yellow	2.5	Circular	White	-ve, rod
CHI-6	White	Yellow	2.5	Circular	White	-ve, rod
CHI-7	White	Yellow	4.0	Circular	White	-ve, rod
CHI-8	White	Yellow	2.8	Circular	White	-ve, rod
CHI-9	White	Yellow	1.5	Circular	White	-ve, rod
CHI-10	White	Yellow	3.0	Circular	White	-ve, rod

CHI-11	White	Yellow	4.0	Circular	White	-ve, rod
CHI-12	White	Yellow	2.5	Circular	White	-ve, rod
CHI-13	White	Yellow	2.0	Circular	White	-ve, rod
CHI-14	White	Yellow	2.7	Circular	White	-ve, rod
CHI-15	White	Yellow	2.8	Circular	White	-ve, rod
CHI-16	White	Yellow	3.0	Circular	White	-ve, rod
CHI-17	White	Yellow	3.5	Circular	White	-ve, rod
CHI-18	White	Yellow	3.0	Circular	White	-ve, rod
CHI-19	White	Yellow	3.5	Circular	White	-ve, rod
CHI-20	White	Yellow	4.0	Circular	White	-ve, rod
KD-1	White	Yellow	4.0	Circular	White	-ve,rod
KD-2	White	Yellow	2.0	Circular	White	-ve,rod
KD-3	White	Yellow	2.5	Circular	White	-ve,rod
KD-4	White	Yellow	3.0	Circular	White	-ve,rod
KD-5	White	Yellow	3.5	Circular	White	-ve,rod
KD-6	White	Yellow	3.6	Circular	White	-ve,rod
KD-7	White	Yellow	3.0	Circular	White	-ve,rod
KD-8	White	Yellow	2.7	Circular	White	-ve,rod
KD-9	White	Yellow	2.9	Circular	White	-ve,rod
KD-10	White	Yellow	3.5	Circular	White	-ve,rod
KD-11	White	Yellow	4.0	Circular	White	-ve,rod
KD-12	White	Yellow	4.0	Circular	White	-ve,rod
KD-13	White	Yellow	3.2	Circular	White	-ve,rod
KD-14	White	Yellow	2.8	Circular	White	-ve,rod
KD-15	White	Yellow	3.4	Circular	White	-ve,rod
KD-16	White	Yellow	4.0	Circular	White	-ve,rod
KD-17	White	Yellow	4.0	Circular	White	-ve,rod

Appendix A: table 2: continue...

KD-18	White	Yellow	3.0	Circular	White	-ve,rod
KD-19	White	Yellow	3.5	Circular	White	-ve,rod
KD-20	White	Yellow	3.6	Circular	White	-ve,rod
LG-1	White	Yellow	4.3	Circular	White	-ve,rod
LG-2	White	Yellow	4.5	Circular	White	-ve,rod
LG-3	White	Yellow	4.2	Circular	White	-ve,rod
LG-4	White	Yellow	4.0	Circular	White	-ve,rod
LG-5	White	Yellow	3.5	Circular	White	-ve,rod
LG-6	White	Yellow	2.0	Circular	White	-ve,rod
LG-7	White	Yellow	1.5	Circular	White	-ve,rod
LG-8	White	Yellow	2.5	Circular	White	-ve,rod
LG-9	White	Yellow	4.5	Circular	White	-ve,rod

LG-10	White	Yellow	2.5	Circular	White	-ve,rod
LG-11	White	Yellow	3.0	Circular	White	-ve,rod
LG-12	White	Yellow	3.3	Circular	White	-ve,rod
LG-13	White	Yellow	3.5	Circular	White	-ve,rod
LG-14	White	Yellow	4.2	Circular	White	-ve,rod
LG-15	White	Yellow	2.0	Circular	White	-ve,rod
LG-16	White	Yellow	1.5	Circular	White	-ve,rod
LG-17	White	Yellow	2.5	Circular	White	-ve,rod

CHI= Chilga, KD= Kimir Dingay, LG=Lay Gayint,-ve= negative

Appendix A: Table 3. Different carbon sources utilized by *Rhizobium* isolates

Isolates	Glucose	Fructose	Xylose	Sucrose	Lactose	Adonitol	Inositol	Sorbitol	Raffinose	Arabinose	Mannose	Glycerol	Dextrose	Rhamnose	Maltose
CHI-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-2	+	+	+	+	_	+	+	+	+	+	+	+	+	+	+
CHI-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-8	+	+	+	+	+	+	+	+	+	_	+	+	+	+	+
CHI-9	+	+	+	+	_	+	+	+	+	+	+	+	+	+	+
CHI-10	+	+	+	+	_	+	+	+	+	+	+	+	+	+	+
CHI-11	+	+	+	+	_	+	+	+	+	_	+	+	+	+	+
CHI-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Appendix A: Table 3: continue....

CHI-15	+	+	+	+	+	+	+	+	+	_	+	+	+	+	+
CHI-16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-18	+	+	+	+	+	+	+	+	+	_	+	+	+	+	+
CHI-19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CHI-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-3	+	+	+	+	+	+	+	+	+	_	+	+	+	+	+
KD-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-7	+	+	+	+	+	+	+	+	+	_	_	+	+	+	+

KD-8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-14	+	+	+	+	+	+	+	+	+	+	_	+	+	+	+
KD-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-18	+	+	+	+	+	+	+	+	+	+	_	+	+	+	+
KD-19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KD-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-4	+	+	+	+	+	+	+	+	+	_	+	+	+	+	+
LG-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-10	+	+	+	+	+	+	+	+	+	_	+	+	+	+	+
LG-11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LG-17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

CHI= Chilga, KD= Kimir Dingay, LG=Lay Gayint**, (+**) = growth and (-) **=**no growth

Appendix A: Table 4. Tolerance of *Rhizobium* isolates at different pH value

ISOLATES	pH 4	pH 4.5	pH 5	pH 5.5	pH 6	pH 8	pH 9
CHI-1	_	_	_	+	+	+	+
CHI-2	_	_	+	+	+	+	_
CHI-3	_	_	+	+	+	+	+
CHI-4	_	_	+	+	+	+	_
CHI-5	_	_	+	+	+	+	_
CHI-6	_	_	_	+	+	+	_
CHI-7	_	_	+	+	+	+	_
CHI-8	_	_	+	+	+	+	_
CHI-9	_	_	+	+	+	+	+
CHI-10	_	_	_	+	+	+	+

CHI-11	_	_	_	+	+	+	_
CHI-12	_	_	+	+	+	+	+
CHI-13	_	_	+	+	+	+	_
CHI-14	_	_	+	+	+	+	+
CHI-15	_	_	+	+	+	+	+
CHI-16	_	_	+	+	+	+	_
CHI-17	_	_	+	+	+	+	_
CHI-18	_	_	+	+	+	+	_
CHI-19	_	_	+	+	+	+	_
CHI-20	_	_	+	+	+	+	+
KD-1	_	_	_	+	+	+	_
KD-2	_	_	+	+	+	+	_
KD-3	_	_	+	+	+	+	+
KD-4	_	_	+	+	+	+	+
KD-5	_	_	+	+	+	+	_
KD-6	_	_	_	+	+	+	_
KD-7	_	_	_	+	+	+	+
KD-8	_	_	+	+	+	+	+
KD-9	_	_	+	+	+	+	_
KD-10	_	_	+	+	+	+	_
KD-11	_	_	+	+	+	+	+
KD-12	_	_	+	+	+	+	_
KD-13	_	_	_	+	+	+	+
KD-14	_	_	+	+	+	+	_
KD-15	_	_	_	+	+	+	_
KD-16	_	_	_	+	+	+	+
KD-17	_	_	+	+	+	+	+
KD-18	_	_	+	+	+	+	_
KD-19	_	_	_	+	+	+	_
KD-20	_	_	+	+	+	+	+
LG-1	_	_	_	+	+	+	+
LG-2	_	_	_	+	+	+	_

Appendix A: Table 4. Continue...

LG-3	_	_	+	+	+	+	+
LG-4	_	_	+	+	+	+	+
LG-5	_	_	_	+	+	+	_
LG-6	_	_	_	+	+	+	_
LG-7	_	_	+	+	+	+	+
LG-8	_	_	_	+	+	+	+
LG-9	_	_	+	+	+	+	_
LG-10	_	_	+	+	+	+	+
LG-11	_	_	+	+	+	+	_
LG-12	_	_	_	+	+	+	_
LG-13	_	_	_	+	+	+	+
LG-14	_	_	+	+	+	+	+

LG-15	_	_	+	+	+	+	_
LG-16	_	_	+	+	+	+	+
LG-17	_	_	+	+	+	+	_

CHI= Chilga, KD= Kimir Dingay, LG=Lay Gayint**, (+**) = growth and (-) **=** no growth

Appendix A: Table 5. Tolerance of *Rhizobium* isolates at different salt concentrations.

ISOLATES	0.1 % (w/v) Nacl	0.5 % (w/v) Nacl	1 % (w/v) Nacl	2 % (w/v) Nacl
CHI-1	_	+	_	_
CHI-2	_	_	+	_
CHI-3	_	+	_	_
CHI-4	_	_	+	+
CHI-5	_	_	+	_
CHI-6	_	_	+	_
CHI-7	_	_	+	_
CHI-8	_	+	_	_
CHI-9	_	+	_	_
CHI-10	_	_	+	_
CHI-11	_	_	+	_
CHI-12	_	_	_	+
CHI-13	_	_	+	_
CHI-14	_	+	_	_
CHI-15	_	+	_	_
CHI-16	_	+	_	_
CHI-17	_	_	+	_
CHI-18	_	_	_	+
CHI-19	_	_	+	_
CHI-20	_	+	_	_
KD-1	+	_	_	_
KD-2	_	+	_	_
KD-3	_	+	_	_
KD-4	+	_	_	_

Appendix A: Table 5. Continue....

KD-5	_	_	+	_
KD-6	_	_	+	_
KD-7	+	_	_	_
KD-8	+	_	_	_
KD-9	+	_	_	_
KD-10	_	_	+	_
KD-11	_	+	_	_
KD-12	_	+	_	_
KD-13	+	_	_	_
KD-14	+	_	_	_
KD-15	+	_	_	_
KD-16	_	+	_	_

KD-17	_	_	+	_
KD-18	_	+	_	_
KD-19	_	+	_	_
KD-20	+	_	_	_
LG-1	_	+	_	_
LG-2	_	+	_	_
LG-3	+	_	_	_
LG-4	_	_	+	_
LG-5	_	+	_	_
LG-6	+	_	_	_
LG-7	_	_	_	+
LG-8	+	_	_	_
LG-9	_	+	_	_
LG-10	_	_	_	+
LG-11	_	_	+	_
LG-12	+	_	_	_
LG-13	_	+	_	_
LG-14	_	_	_	+
LG-15	_	+	_	_
LG-16	_	_	+	_
LG-17	_	+	_	_

CHI= Chilga, KD= Kimir Dingay, LG=Lay Gayint**, (+**) = growth and (-) = no growth

Appendix A: Table 6. Tolerance of *Rhizobium* isolates at different temperature.

ISOLATES	5 ^{0}C	10 ^{0}C	15 ^{0}C	35 ^{0}C	40 ^{0}C	45 ^{0}C
CHI-1	_	_	+	+	+	_
CHI-2	_	_	+	+	+	_
CHI-3	_	+	+	+	+	+
CHI-4	_	_	+	+	+	_
CHI-5	_	_	+	+	+	_
CHI-6	_	_	+	+	+	_
CHI-7	_	_	+	+	+	_
CHI-8	_	_	+	+	+	_
CHI-9	_	+	+	+	+	+
CHI-10	_	_	+	+	+	_

CHI-11	_	+	+	+	+	+
CHI-12	_	_	+	+	+	_
CHI-13	_	+	+	+	+	+
CHI-14	_	_	+	+	+	_
CHI-15	_	_	+	+	+	_
CHI-16	_	_	+	+	+	_
CHI-17	_	+	+	+	+	+
CHI-18	_	+	+	+	+	+
CHI-19	_	_	+	+	+	_
CHI-20	_	_	+	+	+	_
KD-1	+	+	+	+	_	_
KD-2	+	+	+	+	_	_
KD-3	+	+	+	+	+	_
KD-4	+	+	+	+	+	_
KD-5	+	+	+	+	_	_
KD-6	+	+	+	+	_	_
KD-7	+	+	+	+	_	_
KD-8	+	+	+	+	+	_
KD-9	+	+	+	+	_	_
KD-10	+	+	+	+	_	_
KD-11	_	+	+	+	_	_
KD-12	_	+	+	+	_	_
KD-13	+	+	+	+	_	_
KD-14	+	+	+	+	+	_
KD-15	_	+	+	+	_	_
KD-16	_	+	+	+	_	_
KD-17	+	+	+	+	_	_
KD-18	_	+	+	+	_	_
KD-19	_	+	+	+	_	_
KD-20	+	+	+	+	_	_
LG-1	_	+	+	+	_	_
LG-2	_	+	+	+	_	_

Appendix A: Table 6. Continue...

LG-3	+	+	+	+	+	_
LG-4	+	+	+	+	_	_
LG-5	_	+	+	+	_	_
LG-6	_	+	+	+	_	_
LG-7	_	+	+	+	_	_
LG-8	_	+	+	+	_	_
LG-9	_	+	+	+	_	_
LG-10	+	+	+	+	_	_
LG-11	_	+	+	+	_	_
LG-12	_	+	+	+	+	_
LG-13	+	+	+	+	_	_
LG-14	+	+	+	+	_	_

LG-15	_	+	+	+	_	_
LG-16	+	+	+	+	_	_
LG-17	_	+	+	+	_	_

CHI= Chilga, KD= Kimir Dingay, LG=Lay Gayint**, (+)** = growth and (-) **=** no growth

Appendix A: Table 7. Tolerance of *Rhizobium* isolates at different antibiotics with different antibiotics concentrations.

isolates	Erythromycin (µg)				Ampicillin (µg)				Tetracycline (µg)				Chloramphenicol (µg)			
	100	125	150	200	100	125	150	200	5	10	15	25	5	10	15	25
CHI-1	+	+	+	_	+	+	+	_	+	+	_	_	+	+	+	_
CHI-2	+	+	_	_	+	+	+	_	+	+	_	_	+	+	_	_
CHI-3	+	+	+	_	+	+	+	_	+	+	_	_	+	+	+	_
CHI-4	+	+	+	_	+	+	_	_	+	_	_	_	+	+	+	_
CHI-5	+	+	+	_	+	+	_	_	+	_	_	_	+	+	+	_
CHI-6	+	+	_	_	+	+	+	_	+	+	_	_	+	+	+	_
CHI-7	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
CHI-8	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
CHI-9	+	+	+	_	+	+	+	_	+	_	_	_	+	+	_	_
CHI-10	+	+	+	_	+	+	+	_	+	+	_	_	+	+	+	_
CHI-11	+	+	_	_	+	+	+	_	+	+	_	_	+	+	_	_
CHI-12	+	+	+	_	+	+	+	_	+	_	_	_	+	+	_	_
CHI-13	+	+	_	_	+	+	+	_	+	_	_	_	+	+	_	_
CHI-14	+	+	+	_	+	+	+	_	+	_	_	_	+	+	+	_
CHI-15	+	+	_	_	+	+	+	_	+	+	_	_	+	+	_	_
CHI-16	+	+	+	_	+	+	+	_	+	+	_	_	+	+	+	_
CHI-17	+	+	+	_	+	+	+	_	+	+	_	_	+	+	+	_
CHI-18	+	+	+	_	+	+	+	_	+	_	_	_	+	+	+	_
CHI-19	+	+	_	_	+	+	+	_	+	+	_	_	+	+	+	_
CHI-20	+	+	_	_	+	+	+	_	+	_	_	_	+	+	+	_
KD-1	+	+	+	_	+	+	+	_	+	_	_	_	+	+	+	_

Appendix A: Table 7. Continue....

KD-2	+	+	+	_	+	+	_	_	+	+	_	_	+	+	+	_
KD-3	+	+	+	_	+	+	+	_	+	+	_	_	+	+	+	_
KD-4	+	+	_	_	+	+	+	_	+	+	_	_	+	+	_	_
KD-5	+	+	_	_	+	+	+	_	+	_	_	_	+	+	+	_
KD-6	+	+	+	_	+	+	_	_	+	_	_	_	+	+	+	_
KD-7	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
KD-8	+	+	+	_	+	+	+	_	+	+	_	_	+	+	+	_
KD-9	+	+	+	_	+	+	+	_	+	+	_	_	+	+	+	_
KD-10	+	+	+	_	+	+	_	_	+	_	_	_	+	+	+	_
KD-11	+	+	+	_	+	+	+	_	+	+	_		+	+	_	_
KD-12	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
KD-13	+	+	+	_	+	+	+	_	+	_	_	_	+	+	_	_

KD-14	+	+	_	_	+	+	+	_	+	+	_	_	+	+	+	_
KD-15	+	+	_	_	+	+	_	_	+	+	_	_	+	+	+	_
KD-16	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
KD-17	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
KD-18	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
KD-19	+	+	_	_	+	+	+	_	+	_	_	_	+	+	+	_
KD-20	+	+	_	_	+	+	_	_	+	_	_		+	+	+	_
LG-1	+	+	+	_	+	+	_	_	+	_	_	_	+	+	_	_
LG-2	+	+	+	_	+	+	+	_	+	_	_	_	+	+	+	_
LG-3	+	+	_	_	+	+	+	_	+	+	_	_	+	+	_	_
LG-4	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
LG-5	+	+	_	_	+	+	_	_	+	+	_	_	+	+	+	_
LG-6	+	+	_	_	+	+	+	_	+	_	_	_	+	+	+	_
LG-7	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
LG-8	+	+	_	_	+	+	+	_	+	+	_	_	+	+	+	_
LG-9	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
LG-10	+	+	+	_	+	+	+	_	+	+	_	_	+	+	_	_
LG-11	+	+	+	_	+	+	+	_	+	_	_	_	+	+	+	_
LG-12	+	+	+	_	+	+	_	_	+	+	_	_	+	+	+	_
LG-13	+	+	_	_	+	+	_	_	+	+	_	_	+	+	_	_
LG-14	+	+	+	_	+	+	+	_	+	_	_	_	+	+	+	_
LG-15	+	+	+	_	+	+	+	_	+	_	_	_	+	+	+	_
LG-16	+	+	_	_	+	+	_	_	+	+	_	_	+	+	_	_
LG-17	+	+	+	_	+	+	_	_	+	+	_	_	+	+	_	_

CHI= Chilga, KD= Kimir Dingay, LG=Lay Gayint**, (+)** = growth and (-) **=** no growth

Appendix A: Table 8. N-free Nutrient Solution (Broughton and Dilworth, 1970).

Stock Solutions	Element	Final Molarity(μM)	Form	MW	g/l	Molarity of Stock Solution (M)
1	Ca	1000	$CaCl_2 \cdot 2H_20$	147.03	294.1	2.0
2	P	500	KH_2P0_4	136.09	136.1	1.0
3	Fe	10	Fe-Citrate	335.04	6.7	0.02
	Mg	250	$MgSO_4 \cdot 7H_20$	246.5	123.3	0.5
	K	1500	K_2SO_4	174.06	87.0	0.5

	Mn	1	$MnSO_4 \cdot H_20$	169.02	0.338	0.002
4	B	2	H_3BO_4	61.84	0.247	0.004
	Zn	0.5	$ZnSO_4 \cdot 7H_20$	287.56	0.288	0.001
	Cu	0.2	$CuSO_4 \cdot 5H_20$	249.69	0.100	0.004
	Co	0.1	$CoSO_4 \cdot 7H_20$	281.12	0.056	0.0002
	Mo	0.1	$Na_2MoO_4 \cdot 2H_20$	241.98	0.048	0.0002

For each 10 liters of full strength culture solution, take 5.0 ml each of solutions 1 to4, then add to 5.0 liters of water, then dilute to 10 liters. Use 1 N NaOH to adjust the pH to 6.6-6.8. For plus N control treatments, KNO_3 (0.05%) is added giving an N concentration of 70 ppm.

Appendix B: some representative figures taken during collection and identification of Soil sampling.